The effects of cholinergic and dopaminergic neurons on hippocampal learning and memory processes

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Doctor of Philosophy

University of Cambridge
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Churchill College

September 2017

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THE EFFECTS OF CHOLINERGIC AND DOPAMINERGIC NEURONS ON HIPPOCAMPAL LEARNING AND MEMORY PROCESSES

Sze-Man Clara Tang, Churchill College, University of Cambridge
This dissertation is submitted for the degree of Doctor of Philosophy, September 2017

Dysfunction of cholinergic and dopaminergic systems has been implicated in memory function deficits that are core pathology and associated features of several neurological disorders. However, in order to develop more effective treatments, it is crucial to better understand how different aspects of learning and memory are modulated by these neuromodulatory systems. Using optogenetic stimulation or silencing, this thesis aims to investigate the effects of cholinergic and dopaminergic modulation on various hippocamal learning and memory processes.

To understand how these neuromodulatory systems modulate hippocampal network activity, I first examined their effects on hippocampal local field potentials in urethane-anaesthetised mice. I demonstrated that optogenetic cholinergic activation suppressed slow oscillations, shifting brain activity to a state dominated by theta and gamma oscillations. In contrast, dopaminergic activation suppressed gamma oscillations. Second, to directly probe the effects of neuromodulation on different stages of spatial learning, I acutely activated or inactivated cholinergic or dopaminergic neurons during various behavioural tasks. My findings suggested that cholinergic activation, solely during the reward phase of a long-term spatial memory task, slowed place learning, highlighting the importance of temporally-precise neuromodulation. Moreover, dopaminergic stimulation may enhance place learning of a food rewarded task, supporting a role for dopamine in spatial learning. In addition, I tested the effects of cholinergic and dopaminergic modulation on reversal learning and found that cholinergic inactivation and dopaminergic activation appear to impair this process.

Together, these findings emphasise the importance of cholinergic and dopaminergic modulation in learning and memory. They suggest that precise timing of neuromodulator action is critical for optimal learning and memory performance, and that acetylcholine and dopamine support complementary processes that allow for effective learning and adaptation to changing environments.

Acknowledgements

I would like to thank my supervisor Professor Ole Paulsen. In addition, thanks go to Professor Tim Bussey, Dr Lisa Saksida, Dr John Apergis-Schoute, Dr Teemu Aitta-aho and Rachel Spicer for their advice on immunohistochemistry, behavioural testing and statistics. It has been a pleasure to work with all the members of the Neuronal Oscillations Group, past and present; special thanks go to Dr Audrey Hay, Dr Olivia Shipton, Dr Mohamady El-Gaby and Dr James Butler for their guidance on electrophysiology and help with proof-reading this thesis. I would also like to acknowledge members of the Churchill College MCR; I am particularly grateful for their friendship and support throughout the past years. Finally, many thanks go to Churchill College and the Cambridge Trust for providing the funding for my PhD and making this work possible.

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List of abbreviations

ACh	acetylcholine
AChE	acetylcholinesterase
AD	Alzheimer's disease
ANOVA	analysis of variance
AP	anterior-posterior
ArchT	archaerhodopsin-3 (from the *Halorubrum* strain TP009)
CA	*cornu ammonis*
ChAT	choline acetyltransferase
ChR2	channelrhodopsin-2
CNS	central nervous system
DA	dopamine
DAT	dopamine transporter
LFP	local field potential
(t-)LTP	(timing-dependent) long-term potentiation
(t-)LTD	(timing-dependent) long-term depression
mAChR	muscarinic acetylcholine receptor
MS	medial septum
MUA	multi-unit activity
OF	open field
nAChR	nicotinic acetylcholine receptor
PD	Parkinson's disease
PSD	power spectral density
REM	rapid eye movement
SC	Schaffer collateral
SLR	spontaneous location recognition
SO	slow oscillations
STDP	spike-timing dependent plasticity
SWR	sharp-wave-ripple
TH	tyrosine hydroxylase
VTA	ventral tegmental area
WM	working memory
WT	wild-type

Chapter 1

Introduction

Memory is one of the most fundamental mental processes that supports all of our daily functions beyond simple reflexes and stereotyped behaviours; it is largely what makes each human unique. In order to decipher how neuronal networks encode and store memories, and how these processes can fail in disease, it is critical to understand the cellular network mechanisms that underlie and modulate memory formation.

This thesis focuses on the effects of two neuromodulatory (cholinergic and dopaminergic) systems on spatial learning and memory. Therefore, after an overview of the various forms of human cognitive functions, I will introduce spatial memory, a widely used model for understanding the mechanisms underlying cognition, and describe the different navigation strategies that may be employed. Spatial information is thought to be stored and flexibly used for navigation in the hippocampus (Morris et al., 1982; Sutherland et al., 1989; Murray et al., 1998; Ekstrom et al., 2003) and, as such, I will next discuss what is currently known about the hippocampal mechanisms underlying spatial memory formation. Another important aspect for learning is the ability to adapt behaviours to changing rewards and goals called behavioural flexibility (Izquierdo et al., 2017); the neural mechanisms that contribute to this behaviour will also be outlined.

Loss of memory (dementia) not only has distressing personal consequences, it is also a major social and financial burden on society. It is a key symptom in a number of devastating diseases including Alzheimer's disease (AD; Whitehouse et al., 1982; Hippius and Neundörfer, 2003), cognitive impairment associated with schizophrenia (Radhakrishnan et al., 2012; Furth et al., 2013) and Parkinson's disease (PD; Docherty and Burn, 2010). These diseases are typically associated with dysfunction of certain neuromodulatory systems - the cholinergic system in AD and dopaminergic system in schizophrenia and PD, and their modulation has been a focus of treatment strategies and drug development approaches (Birks, 2006; Chaudhuri and Schapira, 2009; Citrome, 2014).

However, the efficacy of these treatments is variable and most only facilitate the alleviation of symptoms or temporarily delay disease progression. Thus, there is still considerable need for better understanding of how these systems and memory processes interact. As such, I will review what recent studies have discovered about the effects of cholinergic and dopaminergic modulation on hippocampal learning and memory processes, before further exploring their effects on hippocampal network oscillations (chapter 3) and performance in spatial short-term (chapter 4) and long-term (chapter 5) memory tasks.

1.1 Memory systems

Memory is defined as the process by which experienced-based information is encoded, stored and retrieved. Firstly, memories can be classified by the duration of information retention, i.e. the duration between memory acquisition and retrieval (Atkinson and Shiffrin, 1968; Baddeley and Warrington, 1970; Milner, 1972). *Short-term memory* is the capacity to temporarily hold a limited amount of information in a very accessible state (Eysenck, 1988). *Working memory* (WM) is a type of short-term memory used to describe when that short-term memory is used and processed for cognitive tasks (Miller, 1956; Baddeley and Hitch, 1974; Becker and Morris, 1999; Cowan, 2008). Deficits in WM are hallmark symptoms in a number of neurodegenerative and neuropsychiatric disorders such as AD (Baddeley et al., 1991), schizophrenia (Goldman-Rakic, 1994) and ADHD (Barkley, 1997).

Information stored in short-term memory can be consolidated and transferred into *long-term memory*, which involves more long-lasting storage, management, and retrieval of information (Dudai, 2004; Wang and Morris, 2010). Long-term memory can be divided according to the content encoded - non-declarative or declarative (Gold and McGaugh, 1975; Anderson, 1976; Squire, 1992).

Non-declarative (or implicit) memories refer to memories that do not require conscious thought, unconsciously recalling previously stored information without awareness of the experiences that have formed them. They are required for executing integrated procedures involving both cognitive and motor skills such as typing or bike-riding. These memories are hippocampus-independent (Schacter and Daniel, 1987). This thesis focuses on *declarative* (or explicit) memories, which are especially susceptible to decline attributable to aging (McIntyre and Craik, 1987), neurodegenerative (Almkvist and Winblad, 1999; Elgh et al., 2009) or psychiatric diseases (Boyer et al., 2007). Declarative memories describe the recollection of information and experiences that can be consciously recalled and verbally described (i.e. declared) such as facts and verbal knowledge. They are known to depend on the hippocampus (Tulving, 2002).

Declarative memory can be further divided into two main branches: (i) *semantic memory* (or conceptual knowledge) represents concept-based memories that are unrelated to specific experiences such as memories of meanings, objects, understandings and facts (Tulving, 1972); (ii) *episodic memory* refers to detailed explicit recollection of events in terms of their time, location, elements, associated emotions, and other contextual who, what and why. These memories are autobiographical and can be used for planning actions. It has been proposed that as related episodes are encoded repeatedly by the self-referenced episodic memory system, knowledge gradually becomes context independent, forming semantic memories (Dere et al., 2006; Ferbinteanu et al., 2006).

While these different forms of complex memories can be studied in humans, whether rodents, the most popular animal models used in cognitive neuroscience, also possess the capacity to process and store complex information similar to human declarative memory is still under debate (Griffiths et al., 1999; Tulving, 2002). A type of memory that can be investigated in rodents, which has been generally accepted to show similarities to semantic and episodic

memories in humans, is spatial memory (O'Keefe and Conway, 1978; Cohen and Eichenbaum, 1994; Burgess et al., 2002; Buzsáki and Moser, 2013). How the cholinergic and dopaminergic systems affect spatial memory in rodents will be the focus of this thesis.

1.1.1 Spatial memory

Spatial memory refers to the storage and retrieval of information about one's surroundings and its spatial orientation, vital for navigation (Morris et al., 1982; Nakazawa et al., 2004). It is one of the most important forms of higher cognitive processing required for survival. As an animal navigates its environment, landmark cues are used to construct a coherent spatial representation of the environment in memory.

Much like the dichotomy of declarative memory, two forms of spatial representation used to guide spatial learning and memory can be distinguished (Tulving, 1972; Burgess, 2006; Suddendorf and Corballis, 2007; Schiller et al., 2015). *Allocentric* or map-based navigation refers to the use of spatial relationships among visible or detectable landmarks in the environment to assist in defining the animal's location, largely independently of how the animal got there; much as semantic memory explicitly defines objects, fact and events independently of temporal context. The spatial metric required to construct this map is believed to result from a second mechanism, referred to as *egocentric* navigation or path integration (Feigenbaum and Rolls, 1991; Klatzky, 1998). Egocentric navigation is based on self-referenced information, in the same way as learning and recalling first-person experiences in episodic memory. During egocentric navigation, the animal defines itself in relation to the objects, goals, or intra-maze locations by actively moving around its environment and keeping track of the distances travelled, and direction of rotations as the animal explores its surroundings (Feigenbaum and Rolls, 1991; Klatzky, 1998). Computing

translocation relative to its starting location allows the animal to return to its starting location using the shortest route. Allocentric and egocentric representation always work in tandem, but the availability of external landmarks may determine which strategy dominates (Squire, 1992; Buzsáki, 2005).

1.2 Neural mechanisms underlying memory trace formation

The formation of new long-term memory traces relies on a two-stage process where novel information is first encoded (or learnt) during alert wakefulness, then consolidated during subsequent rest or sleep (Marr, 1971; Buzsáki, 1989). A wealth of experimental data has shown that the hippocampus plays a critical role in spatial learning and memory, not only in rodents (Morris et al., 1982; Sutherland et al., 1989; Jarrard, 1993), but also in other mammals (Murray et al., 1998) including humans (Ekstrom et al., 2003). Thus, this section will briefly describe the hippocampus before presenting an overview of the hippocampal processes thought to support memory encoding and memory consolidation.

1.2.1 The hippocampus

The hippocampus has long been implicated in the formation of memory since the famous discoveries made from the study of a human patient H.M. H.M.'s medial temporal lobes (including both hippocampi) were surgically removed to treat his epileptic seizures, but as a consequence, he suffered severe anterograde amnesia and his ability to form new memories was lost (Scoville and Milner, 1957). Other patients with bilateral damage to their hippocampi reported similar deficits (Amaral et al., 1986; Jarrard, 1993; Schmolck et al.,

2002). Moreover, pathological changes in the hippocampus can lead to devastating diseases such as AD, the main symptoms of which are cognitive decline, particularly in learning and memory (Fox et al., 1996; Wang et al., 2006). These observations, along with further studies in experimental animals, led to the conclusion that formation of new memories, for facts, place and events, require hippocampal function (Morris et al., 1982; Squire and Zola-Morgan, 1991; Jarrard, 1993). It is thought that subsequent to the initial encoding, the memory trace is eventually stored in another brain region (neocortex) and is then no longer dependent on the hippocampus (Squire and Zola-Morgan, 1991).

The hippocampus has a highly organised lamellar structure, composed of six subregions: the dentate gyrus, hippocampus proper, subiculum, presubiculum, parasubiculum and the entorhinal cortex (Amaral and Witter, 1989). In rodents, the *hippocampus proper* comprises of three parts: *cornu ammonis* (CA)1, CA2 and CA3 (Figure 1.1). The CA3 is especially important, ablation of the CA3 impairs performance in both short-term single trial tasks as well as tasks requiring long-term spatial memory (Sutherland et al., 1983; Brun et al., 2002; Lee and Kesner, 2002; Steffenach et al., 2002; Lee and Kesner, 2003; Kesner, 2007). The CA regions have clearly defined strata: *stratum oriens*, containing the cell bodies of inhibitory basket cells and horizontal trilaminar cells, basal dendrites of pyramidal cells where they receive inputs from other pyramidal neurons, fibres from the medial septum (MS) and commissural fibres from the contralateral hippocampus; *stratum pyramidale* where the cell bodies of pyramidal neurons and interneurons such as axo-axonic cells, bistratified cells and radial trilaminar cells are found; and *stratum radiatum*, containing septal and comissural fibres, much like the *stratum oriens*, importantly, it contains *Schaffer collateral* (SC) fibres, which are projections from CA3 to CA1 (Amaral and Witter, 1995).

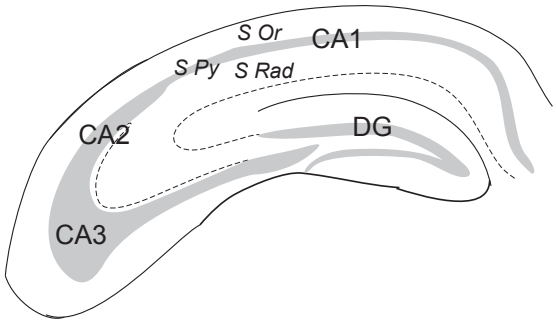


Figure 1.1: The rodent hippocampus. Illustration of the rodent hippocampus outlining the components of the hippocampus proper and dentate gyrus (DG). CA, cornu ammonis; S Or, stratum oriens; S Py, stratum pyramidale; S Rad, stratum radiatum

The rodents' hippocampus exhibits a heterogeneous collection of oscillations of various frequencies. Neural oscillations reflect the summed rhythmic activity of constituent cell populations in the brain (Colgin, 2016). Thus, understanding their patterns, generation mechanisms and functions can provide important insights into network activity and complement single-neuron studies and behavioural assays.

Oscillations are found throughout the central nervous system (CNS) and at all levels, from spike patterns, local field potentials and large-scale oscillations measured by electroencephalography. They can be characterised by oscillation frequency, phase and amplitude and they have been linked to various cognitive functions such as perception, motor control, information transfer and memory (Hebb, 1949; Quiroga, 2013).

Three well characterised types of oscillations can be recorded in the hippocampus: (i) theta, (ii) gamma and (iii) sharp-wave-ripples (SWRs). These oscillations have been shown to contribute to memory encoding and consolidation and may help coordinate the interactions between brain regions (Colgin, 2016). Each of these rhythm bands will be further elaborated in later sections (sections 1.2.2.1 and 1.2.3.3).

1.2.2 Memory encoding

Memory encoding refers to the process during which information is acquired and sensory inputs are processed as memory traces to be stored (Shapiro and Eichenbaum, 1999).

A range of interacting cell types with defined activity profiles encode an animal's spatial coordinates. Electrophysiological recordings from the CA1 and CA3 of freely moving rodents revealed that certain hippocampal pyramidal neurons vary their firing rates depending on the animal's location in its environment (O'Keefe and Dostrovsky, 1971; Ekstrom et al., 2003). Specifically, a particular pyramidal neuron has a certain place in the environment called the cell's *place field* where the pyramidal neuron's firing rate increases. These neurons, discovered by John O'Keefe in the 1970s, were named *place cells* (O'Keefe and Dostrovsky, 1971). Different place cells are active in different places of the environment and collectively, these place cells form an internal neural map of the local environment, providing the brain with a cognitive map - a spatial reference map system (O'Keefe and Nadel, 1978; O'Keefe, 1979; Allen et al., 2012). The hippocampus can contain multiple cognitive maps of different environments represented by different combinations of active place cells. Accordingly, place cells are believed to provide the spatial context for memories and past experiences (Shohamy and Wagner, 2008; Brandon et al., 2014).

Place cells may also have memory functions. In each new environment, determined by external sensory information such as major changes in visuospatial or olfactory cues, the place cells *remap* by changing their preferred firing location and thereby forming a new map (O'Keefe and Conway, 1978; Muller and Kubie, 1987; Bostock et al., 1991; Anderson and Jeffery, 2003). This remapping is learned and, once established, the hippocampal-place-cell representations of those environments remain stable. They persist long-term and transfer to new

environments of the same shape (Lever et al., 2002).

Other spatially-sensitive cells have since been identified. In the 1980s, James Ranck described 'head-direction cells' in the presubiculum, which fire when the animal's head is pointed in a preferred direction and thus providing directional inputs into the place system (Ranck, 1984; Taube et al., 1990). 'Boundary vector cells', found in the subiculum, fire along the specific geometric boundaries of the environment and help with the assessment of distances (Lever et al., 2009). The Moser group described 'grid cells' in the medial entorhinal cortex which are active at regular periodic intervals throughout an environment that together form nodes of an hexagonal grid, thus providing a coordinate system for that particular environment (Hafting et al., 2005). Together, all this information allows for the construction of the spatial metric of the surrounding environment.

1.2.2.1 Theta and gamma rhythms

The activity of hippocampal place cells during active movement is strongly correlated with the *theta rhythm* (Vanderwolf, 1969). During translational motion, the hippocampal local field potential (LFP) is dominated by theta oscillations with a frequency range of 4-12 Hz (Green and Arduini, 1954). Individual theta cycles have been proposed to be a compressed representation of the environment and contribute to memory encoding.

As an animal moves through the place field of a place cell, the place cell will spike earlier and earlier in each succeeding theta cycle (O'Keefe et al., 1993). This advancement of spike times with respect to theta is called *theta phase precession* and has been considered to be a way of temporally compressing the time-scale of trajectory sequences through space into the time-scale for synaptic plasticity (Skaggs et al., 1996; Isaac et al., 2009). Information acquired during learning is believed to be encoded and stored as changes in synaptic weights distributed throughout the network of co-active place cells (Hughes,

1958; Colicos and Syed, 2006; Zenke et al., 2015). Long-term plasticity can be induced via a number of paradigms such as high-frequency stimulation that leads to long-term potentiation (LTP) or low-frequency stimulation that results in long-term depression (LTD; Bliss and Collingridge, 1993; Bear and Malenka, 1994).

Phase precession also provides a model for sequence-retrieval and prediction of the path ahead. Sequential activity among specific hippocampal cell assemblies within one theta cycle is preserved at the level of spike times, suggesting that these theta sequences reflect overlapping recent, current and future positions into single episodes. These theta sequences have been shown to predict goal locations in a value-guided decision-making task (Wikenheiser and Redish, 2015), develop with experience (Feng et al., 2015), and can be modulated by behaviour and various task variables such as length of the path and running speeds (Gupta et al., 2012).

As well as providing a phase code for spatial representation within the hippocampus, theta oscillations may also help coordinate activity between brain regions during sensory information processing. Theta activity coherence has been observed between the hippocampus and prefrontal cortex or the ventral striatum in some spatial memory tasks (Meer and Redish, 2011; Backus et al., 2016; Chen et al., 2016). Theta rhythms are also correlated with rhythmic behaviour such as whisking and sniffing (Kepecs et al., 2006). These synchronisations play an important role in the communication between neural regions and neural plasticity as the hippocampus receives multimodal sensory inputs from various brain regions.

Interestingly, theta rhythms also occur during rapid eye movement (REM) sleep though its function in REM sleep is controversial (Leung, 1984; Hutchison and Rathore, 2015). Some studies have suggested that REM plays an important

role in memory consolidation (Colgin, 2013; Boyce et al., 2016).

Nested within the theta rhythm are often gamma oscillations, characterised as rhythmic activity between 20 and 100 Hz. In the CA1, three classes of gamma oscillations with different frequencies, spectral compositions, spatial distributions and theta phase preferences can be distinguished - slow, medium and fast gamma oscillations generated by the CA3, entorhinal input networks and local CA1 network respectively (Goutagny et al., 2013; Schomburg et al., 2014; Butler and Paulsen, 2015). However, these three distinct types of gamma rhythms appear to serve the same general function. They are thought to act as an index for the gating of information flow through the hippocampus (Colgin et al., 2009).

Coupling between theta and gamma oscillations has been well described in the hippocampus and has been implicated in successful memory encoding. Theta-gamma phase-amplitude coupling increases in rats during a contextual learning task (Tort et al., 2009) and in humans performing working memory and long-term memory tasks (Heusser et al., 2016; Lega et al., 2016). In addition, the power co-modulation of theta and slow gamma rhythm in the hippocampus can predict the performance of rats in a spatial memory task (Shirvalkar et al., 2010). The precision of this cross-frequency coupling can also predict individual working memory performance in humans (Axmacher et al., 2010). This coupling is highly dynamic; its amplitude can be modulated in task-dependent patterns, particularly during decision-making behavioural epochs in a matter of milliseconds (Tort et al., 2008).

1.2.2.2 Mechanisms of theta generation

The MS, which sends the majority of cholinergic inputs to the hippocampus, has long been considered to have an important role in the generation of theta

rhythm. Lesioning or inactivating the septal area in behaving animals disrupts theta rhythms in structures that receive MS projections including the hippocampus and entorhinal cortex (Green and Arduini, 1954; Petsche et al., 1962; Mitchell et al., 1982; Mizumori et al., 1990). In addition, stimulation of septal cholinergic neurons leads to the generation of theta in the hippocampus (Vandecasteele et al., 2014). Transition to theta activity in the MS precedes appearance of theta in the hippocampus by ∼500 ms (Bland et al., 1999), further supporting the idea that theta is initiated by septo-hippocampal projections.

In particular, theta is thought to be paced by septal GABAergic interneurons expressing hyperpolarisation-activated and cyclic nucleotide-gated nonselective cation channels (HCN channels; Varga et al., 2008). They spike rhythmically at theta frequencies and are phase-locked to hippocampal theta. In contrast, septal cholinergic neurons do not spike rhythmically at theta frequencies and are thought to modulate the excitability of other neurons such that their theta rhythmic firing is facilitated (Simon et al., 2006).

Other mechanisms in addition to MS inputs have been proposed to be involved in the generation of hippocampal theta rhythm. Theta rhythms have been recorded *in vitro* in isolated intact hippocampal slices, in the absence of afferent inputs (Goutagny et al., 2009). The rhythms appear spontaneously (without any addition of drugs) and even when the CA3 was removed, theta activity persisted in the CA1. Of note, this study does not exclude the possibility of ACh in inducing theta because while the hippocampus was isolated, ACh fibres may still be present in the hippocampal slices. These fibres can release ACh even though they are no longer connected to the soma any longer. Nevertheless, recordings of CA1 pyramidal neurons and some interneurons revealed that they have different preferred firing frequencies within the theta band (Pike et al., 2000; Zemankovics et al., 2010; Stark et al., 2013) and the interactions between these neurons have been proposed as a potential mechanism that supports intrinsic theta rhythm generation *in vitro*. The medial entorhinal

cortex also generate its own theta rhythm that can affect hippocampal theta activity (Montoya and Sainsbury, 1985). These findings suggest another theory whereby several local brain regions intrinsically generate local theta activity and these different regional generators can be synchronised by weak coupling (for review see Colgin, 2013).

1.2.2.3 Mechanisms of gamma generation

Several models have been postulated to generate gamma oscillations in the hippocampus. I will briefly introduce some of the main theories proposed.

At the local network level, the interneuron-network gamma (ING) model postulates that gamma oscillations are generated via reciprocally connected interneurons alone. The recurrent connectivity of fast, regular-spiking interneurons is enough to entrain and synchronise the entire network to their rhythm (Whittington et al., 1995, 2000). In contrast, the pyramidal-interneuron network gamma (PING) model suggests that the reciprocal feedback circuit of excitatory pyramidal neurons and inhibitory interneurons generate gamma oscillations. Excitatory neurons excite inhibitory interneurons which in turn silence both excitatory and inhibitory neurons. Once the inhibition fades, excitatory neurons spike again, generating the next gamma cycle (Fisahn et al., 1998; Mann et al., 2005; Buzsáki and Wang, 2012).

In addition to these cell types, several receptors have been shown to be necessary for gamma oscillations to occur. Inhibition of fast inhibitory $GABA_A$ receptors blocks gamma oscillations (Whittington et al., 1995; Fisahn et al., 1998; Gillies et al., 2002; LeBeau et al., 2002; Pietersen et al., 2014), while activation of metabotropic glutamate receptors (mGluR), acetlycholine receptors and kainate receptors have been demonstrated to induce *in vitro* gamma oscillations (Whittington et al., 1995; Fisahn et al., 1998; Cunningham et al.,

2003). Furthermore, inhibition of AMPA receptors has been shown to block various models of *in vitro* PING (Gillies et al., 2002; LeBeau et al., 2002; Pietersen et al., 2014), but not ING gamma oscillations (Whittington et al., 1995).

1.2.3 Memory consolidation

For long-term memory, the information encoded must be stabilised, consolidated and maintained over long periods of time. This stabilisation of memories is dependent on the hippocampus, which is thought to integrate information encoded from primary and associative cortical areas into a coherent memory trace, before the memory trace is transferred to and ingrained in hippocampal-neocortex circuits (Maviel et al., 2004; Moscovitch et al., 2006). At least two phases of consolidation have been proposed: cellular and systems consolidation. Exactly how long this consolidation process lasts and whether the memory trace is ever truly independent of the hippocampus is still unclear.

1.2.3.1 Cellular consolidation

Cellular consolidation refers to the cellular process that stabilise encoded information by strengthening the connections between co-active neurons within the hippocampus and other areas (Frankland and Bontempi, 2005; Wang and Morris, 2010). Long-term potentiation (LTP), a form of synaptic plasticity, is the leading cellular model for the initial encoding and subsequent stabilisation of memory. LTP has several features that suggest how memories could be acquired, stored and, later, reliably retrieved. LTP is long-lasting and input-specific, for example, synaptic changes have been recorded to persist for at least six days in freely-moving rats (Doyere et al., 2003). Furthermore, induction of LTP requires precise timing of pre- and post-synaptic activity (Caporale and Dan, 2008), which suggests how this mechanism could form associations between events

in the external world. In particular, it is widely established that most forms of hippocampal plasticity require the activation of NMDA receptors (NMDARs; Collingridge et al., 1983; Bliss and Collingridge, 1993), and NMDAR-dependent synaptic plasticity contributes to the formation of associative, long-term spatial memories (Martin et al., 2000). However, there is yet to be causative evidence that synaptic plasticity mediates learning.

Some strong circumstantial evidence have suggested that LTP indeed contribute to associative spatial learning. Some of the first evidence was contributed by Morris et al. (1986), a study in which they demonstrated that intraventricular infusions of a NMDAR antagonist in rats impaired both NMDAR-dependent LTP in the hippocampus and the ability to learn a spatial location across a number of trials in the Morris water maze task. Subsequently, Tsien et al. (1996) showed that genetic removal of NMDARs in CA1 pyramidal cells of the mouse hippocampus, though not without some extrahippocampal removal, blocked LTP at the CA3-CA1 synapse and impaired spatial associative learning, suggesting that NMDAR-, activity-dependent modifications of CA1 synapses play an essential role in the acquisition of spatial memories. In addition, learning has also been shown to induce LTP (Whitlock et al., 2006; Valenzuela-Harrington et al., 2007) and that acquisition of an associative behavioural task and LTP can both occlude each other (Gruart et al., 2006; Whitlock et al., 2006), further suggesting that the same mechanisms may underpin both.

However, there remains debate as to the exact role of long-term plasticity in long-term spatial memory. Bannerman et al. (2012) found that genetic ablation of NMDARs on dentate gyrus granule cells and dorsal CA1 pyramidal neurons did not affect hippocampus-dependent, spatial reference memory when *Grin1*$^{\Delta DGCA1}$ mice were tested on the Morris water maze despite the loss of NMDAR currents and LTP in dorsal CA1 pyramidal neurons. Rather, *Grin1*$^{\Delta DGCA1}$ mice were impaired at using spatial information to select between competing or overlapping long-term memories. Their study supports the idea

that the hippocampus has an important function in pattern separation required to disambiguate overlapping inputs (Marr, 1971; Rolls and Treves, 1998; Sahay et al., 2011). Thus, there is a need for further investigation into the precise physiological role of hippocampal synaptic plasticity in the encoding and storing of long-term, associative spatial memories.

1.2.3.2 Systems consolidation

The hippocampus is believed to play a time-limited role in declarative memory storage and systems consolidation is required to gradually integrate new memories with pre-existing memories and become independent of the hippocampus (Alvarez et al., 1995; Baddeley et al., 2000). Stabilisation of memories is thought to occur through hippocampally driven reactivation of previously encoded information (i.e. place cell sequences), a process proposed to facilitate the transfer and storage of the memory in the neocortex (O'Neill et al., 2010). The mechanisms that underlie this process require several characteristics. First, reactivation of previously encoded assemblies must repeatedly reactivate independent of behavioural stimuli. Second, the mechanisms must persist for some time after exploration to promote the stabilisation of memory traces in the process of consolidation. Lastly, mechanisms underlying consolidation would need to support plasticity in distributed neocortical circuits so memories that were initially encoded in the hippocampus are gradually stored as changes in synaptic weights in more distributed circuits (Wilson and McNaughton, 1994; Kruskal et al., 2013).

A leading candidate thought to facilitate memory consolidation is the reactivation of stored hippocampal sequences during SWRs (Carr et al., 2011; Girardeau and Zugaro, 2011). The entire sequence of previously active place cells during exploration are reactivated in the same order (*forward replay*) during sleep SWR events (Lee et al., 2002; Csicsvari et al., 2007; Diba and Buzsáki, 2007; Davidson et al., 2009; Gupta et al., 2010). Much like in theta

phase precession, these replayed sequences are temporally compressed into the time-scale for synaptic plasticity (Nádasdy et al., 1999; Lee et al., 2002; Davidson et al., 2009). Interestingly, during SWR events occurring during awake state, in pauses during locomotion (e.g. consummatory behaviour), the place cell sequence can be reactivated temporally forward, in the same sequence as experienced, or reversed, in the opposite order (*reverse replay*; Foster and Wilson, 2006; Csicsvari et al., 2007; Diba and Buzsáki, 2007; Davidson et al., 2009; Gupta et al., 2010). The importance of SWRs in learning and memory is highlighted in studies where disruption of SWRs with electrical stimulation lead to deficits in spatial memory task performance (Girardeau et al., 2009; Ego-Stengel and Wilson, 2010).

1.2.3.3 Sharp wave ripples in spatial learning

During slow wave sleep, waking immobility and also during consummatory behaviour, grooming and brief rest intervals in locomotion, the hippocampus displays large amplitude, transient LFP patterns at an occurrence rate ranging from 0.01 to 2 Hz. Synchronous bursts of CA3 activity lead to large endogenous, non-rhythmic depolarisation of CA1 pyramidal neurons in the stratum radiatum, forming *sharp waves*. Concurrently, interneurons in the CA1 are also widely recruited and fire synchronously at a faster population frequency of 150–250 Hz. This interaction between the inhibition by CA1 interneurons and strong depolarisation from CA3 leads to fast field oscillations called *ripples* (150–250 Hz; Buzsáki et al., 1992; Csicsvari et al., 2000; Brunel and Wang, 2003; Schlingloff et al., 2014).

A number of studies have suggested that SWR events support memory consolidation during sleep (Marr, 1971; Stickgold et al., 2001; O'Neill et al., 2006; Diekelmann and Born, 2010). First, the burst of ripple frequency activity is ideal to provide strong, high frequency stimulation required for the induction of synaptic modifications in downstream neurons via LTP (Bliss and Collingridge,

1993; King et al., 1999; Nádasdy et al., 1999; O'Neill et al., 2006). Induction of hippocampal LTP can remap existing place fields, abolish or create new place fields and rearrange the temporal relationships within the affected cellular population (Dragoi et al., 2003; Dragoi, 2013).

Second, SWR reactivation of newly acquired goal locations is required for the stabilisation of place fields and can predict memory task performance (Dupret et al., 2010). During reactivation, cells that encode nearby place fields that were weakly potentiated during exploration are activated together again with high temporal synchrony, enabling synaptic potentiation and hence promote the strengthening of cell assembles coding nearby locations (King et al., 1999; Frank et al., 2004; O'Neill et al., 2008). Stabilisation of these cognitive maps is thought to allow more consistent activation of place cell assemblies and hence more accurate coding of environments (Roux et al., 2017). It may also help pattern completion of processes when only partial or ambiguous sensory information is available (Carr et al., 2011).

Third, SWR events have also been shown to help refine the cognitive map. Animals that were repeatedly exposed to environments with comparable geometric layouts eventually encode these environments as separate maps (Lever et al., 2002; Leutgeb et al., 2005). It was suggested that reactivation strengthens strongly associated cell assemblies, while simultaneously weakening the joint activities of cells that belong to other assemblies, and thus facilitating the plastic processes that lead to the divergence of cognitive maps (O'Neill et al., 2008; Csicsvari and Dupret, 2013). Sleep may also facilitate the interactions of cell assemblies encoding different environments. While most recently explored environments are most strongly reactivated, other environments the animal might have visited, or will visit, are also reactivated. As a result, this may provide a mechanism for a wider consolidation process, allowing for different experiences to be compared (Carr et al., 2011; Csicsvari and Dupret, 2013).

Notably, the transfer of reactivated activity to extra-hippocampal locations during memory consolidation is also important. SWR events require the coordinated activity of a large number of neurons and this network state has been suggested to be optimal for the transfer of memory traces to extra-hippocampal regions (Buzsáki, 1989; McClelland et al., 1995). Hippocampal activity is heavily interrelated with other regions such as the entorhinal cortex (EC). In fact, normal activation of grid cells in the EC requires excitatory drive from hippocampal place cells (Bonnevie et al., 2013). Consequently, the reactivation of hippocampal place cell assemblies could potentiate the interrelated assemblies between the EC and the hippocampus, potentially playing a role in the fine-tuning of grid cell firing fields as the animal becomes more familiar with the environment (Barry et al., 2012). Moreover, cortical and hippocampal sequences that reflect the same experiences have been shown to replay together during slow wave sleep (Ji and Wilson, 2007). Prefrontal neurons also always fire within 100 ms after hippocampal pyramidal neurons during SWRs and this has been suggested to drive plasticity between pairs of neurons in these two different regions (Wierzynski et al., 2009).

1.2.3.4 Sharp-wave ripples in goal-directed learning

SWRs also occur during waking exploratory periods and these SWR events have been suggested to strengthen place cell representation. Indeed, recent data provided direct evidence that disrupting SWRs during learning prevented the stabilisation and refinement of hippocampal maps (Roux et al., 2017). These SWRs can be influenced by concurrent place-related sensory inputs and those that occur during short immobility intervals are affected by the present location of the animal (O'Neill et al., 2010; Carr et al., 2011). These SWRs could be used to highlight particular locations of behavioural relevance, such as reward locations (Singer and Frank, 2009). In contrast, SWR events that occur during longer immobility periods are not influenced by the animal's location (O'Neill

et al., 2006).

To further this idea, many studies have suggested that place maps remap as animals learn locations of importance. Place cell assemblies reorganise to represent recently learnt goal locations such as the location of the escape platform in an annular Morris water maze task (Hollup et al., 2001) or reward locations on the cheeseboard maze (Dupret et al., 2010). Interestingly, when goal locations were signposted by spatial cues, remapping of place fields does not occur, indicating that this goal-oriented place field reorganisation occurs during allocentric learning context only (Dupret et al., 2010). This remapping could allow for the animal to navigate more efficiently to goals and may be a crucial part of goal-related memory traces (McKenzie et al., 2013).

As previously mentioned, the importance of SWRs in spatial goal learning was demonstrated in spatial memory task where disruption of SWRs, by electrical stimulation in subsequent sleep sessions, impaired the animal's performance (Girardeau et al., 2009; Ego-Stengel and Wilson, 2010). Newly acquired goal locations are reactivated during most SWRs. Notably, how frequently that particular goal location is reactivated during sleep is predictive of subsequent memory performance (Dupret et al., 2010). This is consistent with other findings showing enhanced activity in hippocampal CA3 cells during SWRs following reward, binding reward to experiences that lead up to it (Singer and Frank, 2009). The strength of SWR network synchronisation is also predictive of memory performances in the future (Cheng and Frank, 2008).

Furthermore, recent data have built on these results showing that both increases and decreases in the magnitude of reward are reflected by increases and decreases in the incidence of SWRs, respectively (Ambrose et al., 2016). Interestingly, only reverse replay during awake SWRs are modulated with reward magnitude. At reward locations, both forward and reverse replay can be observed but only reverse replay increased their rate with increased reward and

their rate decreased with decreased reward. In contrast, rate of forward replay remained the same with varying reward level, suggesting a distinction in roles for forward and reverse replay. However, it is debated whether it is actually the network activity of SWR that is important for consolidation or the reactivation of place cells during SWRs itself that is crucial for spatial memory consolidation.

Another study has proposed that SWRs are only enhanced when new locations are learned (Cheng and Frank, 2008; Dupret et al., 2010). Cells fire more actively when the animal receives reward at a newly learned goal location when compared to receiving reward at a location that is familiar to the animal. However, enhanced activation of cells at SWRs is not required for learning but rather may be required for remembering the locations at a later trial. When stabilisation of the cognitive map is blocked with the blockade of NMDA receptors (Kentros et al., 1998; Agnihotri et al., 2004; Dupret et al., 2010), animals showed no impairment in learning reward locations but after a lengthy delay period (more than 1 hour), these animals failed to recall the newly learned locations.

Rapid eye movement (REM) sleep has also been implicated in memory consolidation. During REM sleep, theta rhythms dominate and theta may also play a role in memory consolidation. However, this concept is controversial (Vertes, 2004) and will not be a focus of this thesis.

1.3 Neural basis of behavioural flexibility

In addition to simply learning a response or strategy that achieves a goal or reward, when reward contingencies change, it is arguably just as important to be able to adjust behaviours that continue to maximise reward. This ability to adapt behaviour to changing conditions is referred to as *behavioural flexibility* (Izquierdo et al., 2017). It is disrupted in a number of psychiatric and

neurological disorders such as substance abuse, schizophrenia and PD and, as such, determining the neural basis underlying cognitive flexibility is critical for the understanding of the pathophysiology of these disorders and potentially developing treatments (Swainson et al., 2000; Remijnse et al., 2006; Leeson et al., 2009; Izquierdo and Jentsch, 2012).

One of the most widely used behavioural tests of behavioural flexibility is reversal learning (Izquierdo et al., 2017). In a reversal learning task, subjects are first trained to discriminate and respond differentially to two stimuli, one of which is rewarded every time it is chosen and the other which is not. After all subjects achieve the initial learning and meet a performance criterion, the reward values of the two stimuli are reversed and subjects must then learn to change their behaviour. The subjects are required to flexibly adjust their behaviour when reward contingencies are reversed (Clark et al., 2004; Izquierdo and Jentsch, 2012; Costa et al., 2015).

1.3.1 Cellular and network mechanisms involved in behavioural flexibility

At the brain network level, it is well established that new memories are encoded and stored as changes in synaptic weights distributed throughout the network, resulting in a persistent increase (LTP) in synaptic efficacy. A persistent decrease (LTD) in synaptic weights is arguably just as important for memory storage as bidirectional changes in synaptic strength are necessary to guard the network from the saturating effects of LTP alone (Martin and Morris, 2002; Rosenzweig et al., 2002).

At the cellular level, unchecked potentiation of synapses in an individual cell could stop the neuron from taking part in any further information storage and neurons are thought to tackle this problem by synaptic scaling (Turrigiano and

Nelson, 2004). At the network level, potentiated synapses formed in previous memory traces may act as "noise" and could interfere with the storage of subsequent memory traces in distinct or overlapping groups of synapses.

Several observations have suggested that LTD plays an important role in hippocampus-dependent learning and memory processes and is required to enhance the "signal-to-noise ratio" between the desired potentiated synapses involved in a memory trace and the non-potentiated synapses that do not. First, novel spatial exploration reversed LTP induced *in vivo* (Xu et al., 1998). Second, acquisition of novel spatial information enhanced induction of LTD (Kemp and Manahan-Vaughan, 2004; Manahan-Vaughan and Braunewell, 1999). Third, application of NMDA receptor (NMDAR) antagonists that reduced NMDAR-LTD preserved LTP *in vivo* and enhanced retention of spatial memory (Villarreal et al., 2002). Impaired NMDAR-LTD has also been associated with reversal learning deficit in the Morris water maze as well as a delayed nonmatch to place T-maze task (Nicholls et al., 2008), suggesting that LTD is needed for behavioural flexibility and may serve to weaken earlier encoded memory traces when new information is acquired.

1.4 Neuromodulation for learning and memory

Neuromodulators such as acetylcholine (ACh) and dopamine (DA) have long been recognised to be important for memory function and spatial navigation (Fadda et al., 1996; El-Ghundi et al., 1999; Fadda et al., 2000; Hasselmo, 2006; Cassel et al., 2007; Ragozzino et al., 2009; Adamantidis et al., 2011; Kempadoo et al., 2016). Understanding the hippocampal learning and memory processes that ACh and DA modulate at network and behaviour levels is the main topic of this thesis.

The hippocampus receives constant inputs regarding the behavioural state of the animal, some of which are neuromodulatory inputs that can dramatically alter the functional output of a neural circuit. ACh and DA are of particular interest due to their importance in hippocampal processing during spatial learning and memory. During exploration, ACh levels are high (Giovannini et al., 1998; Fadda et al., 2000; Hironaka et al., 2001; Fadel, 2011), which have been postulated to help encoding of novel information and favour the influence of external inputs compared to intrinsic activity (Hasselmo and Mcgaughy, 2004). In contrast, during subsequent sleep, cholinergic tone is low, which is thought to facilitate consolidation and retrieval of familiar information (Hasselmo, 1999). In goal-directed learning, rewards are associated with increased dopaminergic tone (Goto and Grace, 2005; Grace et al., 2007; Ikemoto, 2007).

Several preclinical and clinical studies have shown that the dopaminergic and cholinergic systems operate in a dynamic balance and disruption of these systems are associated with major neurological and psychiatric disorders such as AD, PD, schizophrenia and drug addiction (Zhang et al., 2004; Chinta and Andersen, 2005; Rolls et al., 2008; Martorana et al., 2010). Restoring the balance of these neurotransmitter systems has been the focus for treating such neuropsychiatric disorders and improving the associated symptoms.

1.5 Acetylcholine: Roles in learning and memory

In the CNS, ACh is an important neuromodulator for motivation, arousal, attention and memory. Since 1906, when Alois Alzheimer first described the symptomatology of the disease that bears his name (Alzheimer, 1906; Hippius and Neundörfer, 2003), cholinergic signalling in the CNS has been of special interest for many neuroscientists. Along with the formation of senile plaques by β-amyloid deposits and the development of neurofibrillary tangles, an alteration in ACh synthesis (i.e. reduced ACh release and up-take) associated with a

marked loss of cholinergic neurons in the basal forebrain are hallmarks of AD (Bartus et al., 1982; Auld et al., 2002).

The cholinergic hypothesis, which postulates that a possible cause of cognitive impairments associated with neurological disorders, especially in AD, is the degeneration of cholinergic neurons, was proposed in the 1970s and 1980s when influential studies found loss of cholinergic markers in the cortex and degeneration of neurons in the basel forebrain of AD patients (Bartus et al., 1982). Indeed, cholinergic abnormalities, including changes in choline transport, cholinergic signalling, ACh release, ACh receptor expression and neurotrophin support, have all been implicated in disorders of attention and cognitive control (Deutsch, 1971; Wilcock et al., 1982; Decker and McGaugh, 1991; Ballinger et al., 2016). Furthermore, multiple groups showed that administration of ACh receptor antagonists could recapitulate the memory deficits observed in AD patients (Muir et al., 1992; Rogers and Kesner, 2004). Importantly, procholinergic drugs have been reported to improve cognition in normal and diseased animals, in healthy volunteers and patients with AD (Levin, 1992; Rusted et al., 1995; Newhouse et al., 1988; Drachman, 1974). Consequently, the majority of treatment strategies and drug development approaches to treat cognitive loss are cholinergic based and intend to treat acetylcholine deficiency.

However, these treatments have had varying efficacy and they only facilitate the alleviation of symptoms or temporarily delay disease progression (Ritchie et al., 2004; Forchetti, 2005; Birks, 2006; Geldmacher et al., 2006; Schmitt and Wichems, 2006; Raina et al., 2008; Rountree et al., 2009). There remains active debate about the validity of the cholinergic hypothesis of cognitive impairment (Martorana et al., 2010). Some reports have suggested that patients with mild cognitive impairment (MCI, a prodromal stage of AD), and early stage AD show no apparent cholinergic neurodegeneration and little changes in choline acetyltransferase and/or acethylcholinesterase activity. Cholinergic systems

may even be upregulated in MCI patients (DeKosky et al., 2002).

The following sections will discuss recent findings on the role of cholinergic circuits in learning and memory. While accumulating evidence points to the importance of cholinergic signalling in hippocampal-dependent learning and memory at the cellular, synaptic, network and behavioural levels, much of our knowledge on cholinergic modulation to date has been derived from exogenous application of receptor agonists or blockers. The precise timings of cholinergic action on hippocampal learning and memory processes remain poorly understood. Specifically, few studies have directly examined the effects of cholinergic activation/inactivation on hippocampal oscillations and its effects on various phases of spatial learning at the behavioural level.

1.5.1 Cholinergic neurons and acetylcholine receptors

The cholinergic system is made up of groups of neurons that use ACh as their neurotransmitter, a number of areas that expresses ACh receptors (muscarinic and nicotinic receptors) and ACh generating projection neurons. ACh is synthesised by choline acetyltransferase (ChAT), which catalyses the transfer of an acetyl group from the coenzyme acetyl-CoA to a natural amine found in the lipid bilayer of the cell membrane (Houser et al., 1983; Oda, 1999).

1.5.1.1 Muscarinic and nicotinic acetylcholine receptors

Activation of the cholinergic system in the CNS is mediated by two classes of receptors: metabotropic muscarinic ACh receptors (mAChRs) and ionotropic nicotinic ACh receptors (nAChRs), both of which are widely distributed throughout the brain (Gotti et al., 2006; Thiele, 2013).

mAChRs are metabotropic receptors that bind ACh and signal via activation of G proteins that in turn open, close and/or influence the kinetics of (mostly)

K^+, Ca^{2+} and non-selective cation channels. There are 5 subtypes of mAChRs that are either selectively linked to G_q proteins (group 1 mAChRs: M_1, M_3, and M_5 subtypes) which activate phospholipase C, or they are selectively coupled to $G_{i/o}$ proteins (group 2 mAChRs: M_2 and M_4 subtypes) that are negatively coupled to adenylate cyclase (reviewed in Wess, 2003), linking ACh activity to a range of biochemical signalling cascades. Moreover, mAChRs are found at pre- and post-synaptic sites throughout the brain and, as a result, activation of mAChRs can have diverse consequences. For example, M_1/M_5 receptors can stimulate the release of DA from striatal synaptosomes and presynaptic M_2/M_4 receptors can act as inhibitory autoreceptors on cholinergic terminals (Douglas et al., 2002) and reduce glutamate release from cortico-cortical and striatal synapses (Higley et al., 2009).

nAChRs function as nonselective, excitatory cation channels, consisting of different combinations of α and β subunits (Picciotto et al., 2001). Their roles are diverse, depending on their subtypes and brain region (Mansvelder et al., 2002). Different nAChR subtypes can mediate release of DA (Grady et al., 2001) and have been shown to contribute to synaptic plasticity in the ventral tegmental area (VTA; Mansvelder and Mcgehee, 2000; Wooltorton et al., 2003), hippocampus (Ge and Dani, 2005; Ji et al., 2001) and prefrontal cortex (Couey et al., 2007). nAChRs have also been reported to play important roles in synchronising neuronal activity (Bucher and Goaillard, 2011).

1.5.1.2 Cholinergic neurons and their projections

Cholinergic cells are commonly classified into six groups with different projection areas (Mesulam, Mufson, Levey and Wainer, 1983; Mesulam, Mufson, Wainer and Levey, 1983). The first group (Ch1) is made up of the cholinergic cells in the MS, which are distinguished from neurons in the vertical limb of the diagonal band of Broca (vDB; Ch2). These two groups are part of the basal forebrain cholinergic complex and provide the major source of

cholinergic fibres to the hippocampus, via the stratum oriens (Auld et al., 2002).

The third group of cholinergic cells (Ch3) are found in the horizontal limb of the diagonal band of Broca (hDB) and they project to both the olfactory bulb and thalamic reticular region. A large group of cells located in the nucleus basalis, preoptic magnocellular nucleus and some parts of the hDB form the fourth group (Ch4), which innervates the neocortex and amygdala. Finally, two midbrain regions (Ch5 and Ch6), composed of the pedunculopontine and laterodorsal tegmentum project mainly to the cortex and various thalamic regions (Mesulam, Mufson, Levey and Wainer, 1983; Rye et al., 1987).

This thesis will focus on the role of ACh in spatial learning and memory, particularly focusing on Ch1, the septo-hippocampal system which is composed of the septal area, the hippocampus, their interconnections and the afferent and efferent pathways that connect them to other brain regions. The septum and the hippocampus are connected mostly by the fimbria and the dorsal fornix bundles.

1.5.2 Cholinergic signalling related to memory

The septo-hippocampal system is unquestionably critical for the formation of spatial memories. During performance of various memory tasks, microdialysis measurements of ACh levels have consistently shown increased ACh efflux in the hippocampus (Stanley et al., 2012; Mitsushima et al., 2013; Roland et al., 2014). A plethora of studies have also revealed the important roles of exogenous and endogenous ACh on hippocampal plasticity and performance in spatial memory tasks (Gu and Yakel, 2011; Cobb and Davies, 2005; Kutlu and Gould, 2016).

1.5.2.1 Cholinergic regulation of synaptic transmission and plasticity

In the hippocampus, ACh modulates neurotransmission in a cell type- and pathway-specific manner. *In vitro* studies have demonstrated that nAChRs and mAChRs on hippocampal glutamatergic and GABAergic neurons can enhance or suppress excitatory or inhibitory transmission (Hasselmo et al., 1995; Dasari and Gulledge, 2011; Tang et al., 2011; Radcliffe et al., 1999; Alkondon and Albuquerque, 2001). Cholinergic activity may also alter the firing properties of CA1 and CA3 pyramidal neurons and thus changing correlated spike activity during rhythmic network oscillations and, as a result, the induction of synaptic plasticity.

As briefly mentioned previously, a wealth of studies has revealed important roles for both mAChRs and nAChRs in hippocampal synaptic plasticity, providing a potential cellular mechanism by which ACh can modulate memory. These effects are thought to be mediated through intracellular signalling pathways downstream of the receptors. Exogenous application or endogenous release of ACh has been shown to induce hippocampal synaptic plasticity in a dose dependent manner; weak activation of mAChR (via application of 0.2-0.5 μM of the muscarinic agonist carbachol) led to induction of muscarinic LTP while strong activation of mAChR (via application of 2-5 μM of carbacol) led to LTD (Auerbach and Segal, 1994, 1996; Markram and Segal, 1990; Scheiderer, 2006; Fernandez de Sevilla et al., 2008; Jo et al., 2010; Ovsepian et al., 2004). Endogenous release of ACh can also induce synaptic plasticity. The type of synaptic plasticity induced is affected by ACh concentration, timing of ACh release, exposure time and the temporal sequence of nAChRs and mAChRs activation in relation to glutamatergic inputs to the CA1 (Fujii and Sumikawa, 2001; Ge and Dani, 2005; Gu et al., 2012; Gu and Yakel, 2011).

Apart from directly causing synaptic plasticity, activation of ACh receptors can also modulate the induction of synaptic plasticity (Shimoshige et al., 1997;

Leung et al., 2003; Ovsepian et al., 2004; Ge and Dani, 2005; Shinoe, 2005). Spike-timing dependent synaptic plasticity (STDP) can be modulated by neuromodulatory inputs before, during and after its induction during normal development and experience (Abraham, 2008; Zhang et al., 2009; He et al., 2015; Brzosko et al., 2017). The type and strength (defined as % change in synaptic weights) of STDP expressed is highly dependent on the relative spike timings of the pre- and post-synaptic inputs during the induction protocol (Markram et al., 1997; Bi and Poo, 1998; Debanne et al., 1998; Song et al., 2000). At the SC-CA1 pathway, pre-before-post spike pairings induce timing-dependent long-term potentiation (t-LTP) while post-before-pre pairings induce timing-dependent depression (t-LTD). Recent data showed that exogenous application of a high concentration of ACh (1 μM) before and during the pre-before-post pairing induction protocol changed t-LTP (in control conditions) to t-LTD (Brzosko et al., 2017). This ACh-facilitated t-LTD is blocked by application of atropine, an antagonist at mAChRs, suggesting that this cholinergic facilitation of t-LTD is mediated by activation of mAChRs. This data implies that activation of mAChRs during coordinated spiking activity biases STDP towards synaptic depression at the SC-CA1 pathway.

The heterogeneous effects of cholinergic modulation on synaptic plasticity are thought to arise from several different mechanisms. Inhibition of small conductance potassium channels via M_1 receptors that in turn enhances NMDA receptor function has been shown to facilitate LTP induction (Markram and Segal, 1990; Buchanan et al., 2010). nAChRs were demonstrated to enhance postsynaptic excitability and facilitate LTP or LTD depending on the timing of ACh application (Ge and Dani, 2005). Presynaptic nAChRs have also been found to modulate GABAergic inhibition of pyramidal neurons (Shimoshige et al., 1997; Ji et al., 2001) and, as such, modulate synaptic plasticity induction at excitatory synapses.

1.5.2.2 Cholinergic effects on rhythmic network oscillations

As alluded to in section 1.2.2.2, the septo-hippocampal network, comprised of the MS local networks, hippocampus and their reciprocal connections, is important for the generation of hippocampal rhythmic oscillations. Cholinergic signaling has been demonstrated to induce theta and gamma oscillations and modulate their strength.

In support of their essential role in facilitating memory encoding, cholinergic signaling is crucial for the regulation and maintenance of theta oscillations as well as memory encoding. Inhibition of mAChRs has been demonstrated to shift the firing of CA1 pyramidal cells towards the theta trough (and as such, away from the encoding peak) during exploration and impair encoding of the environment (Douchamps et al., 2013; Newman et al., 2014). Moreover, theta oscillations in the CA3 are particularly dependent on nAChR signaling, nAChR antagonists abolish theta oscillations in the CA3 (Lu and Henderson, 2010).

While important for theta oscillations, cholinergic neurons in the MS do not appear to actually generate and pace theta oscillations. Theta activity remained following selective lesions of septo-hippocampal cholinergic neurons but the amplitude was significantly reduced. Activity of septal cholinergic neurons have been classified as slow-firing (∼5 Hz), especially when compared to the fast and burst-firing (∼10–18 Hz) GABAergic neurons and cluster-firing (∼8–14 Hz) glutamatergic cells (Serafin et al., 1996; Sotty et al., 2003; Simon et al., 2006). The slow firing rates and the long duration of their after hyperpolarisation indicate that septal cholinergic neurons are limited in their capacity to burst fire theta-related rhythmic activity. Furthermore, several *in vivo* studies found conflicting data as to whether cholinergic neurons increase their spike activity during hippocampal theta activity (Freund and Antal, 1988; Serafin et al., 1996; Sotty et al., 2003; Simon et al., 2006; Hangya et al., 2009). Consequently, it has been proposed that while cholinergic neurons play a role in hippocampal theta

oscillations, septal burst-firing GABAergic cells are the main players in pacing the activity of hippocampal GABAergic interneurons and indirectly, hippocampal pyramidal cells and thereby generating and maintaining hippocampal theta oscillations. Slow-firing cholinergic neurons are important for the regulation and modulation of the amplitude of theta.

Gamma oscillations are also thought to be dependent on cholinergic signalling. They can be induced *in vitro* by activation of muscarinic ACh receptors (Fisahn et al., 1998), kainate receptors (Hajos et al., 2004), or metabotropic glutamate receptors (Whittington et al., 1995; Pálhalmi et al., 2004). Inhibition of cholinergic signaling reduces phase coupling between theta and gamma in the medial entorhinal cortex (Colgin et al., 2009) and reduces encoding of novel environments (Douchamps et al., 2013). Activation of nAChRs has also been demonstrated to induce long-term oscillation in the gamma frequency band, which could facilitate the improvement of cognitive deficits in AD and schizophrenia (Zhang et al., 2015).

Interestingly, recent studies have also shown that activation of mAChRs *in vitro* or *in vivo* optogenetic stimulation of septal cholinergic neurons in awake and anaesthesised animals suppresses SWRs (Vandecasteele et al., 2014).

1.5.2.3 The cholinergic system and spatial learning

Given the shrinkage and degeneration of basal forebrain neurons as well as the reduction of cholinergic markers in cortical and hippocampal areas observed in AD patients, numerous research groups have performed lesion studies (by region-specific radiofrequency and neurotoxic lesions) of the basal forebrain area. As expected, lesions of the basal forebrain led to impairments in learning and memory (Perry et al., 2001; Kitabatake et al., 2003; Rastogi et al., 2014; Ballinger et al., 2016). In particular, immunotoxic lesions of septo-hippocampal cholinergic neurons impaired remapping of distinct environments (Ikonen et al.,

2002). However, there is much debate about the role of the septo-hippocampal cholienrgic input in spatial learning.

There has been conflicting data that questioned the role of the septo-hippocampal projections in spatial memory. Aspiration or electrolytic lesions of the Ch1 has also been shown to hinder acquisition and maintenance of spatial memory in T-maze, water maze and radial arm maze tasks (Hagan et al., 1988; Fraser et al., 1991; Decker et al., 1994; Walsh et al., 1996; Brandner and Schenk, 1998; Janis et al., 1998; Johnson et al., 2002; van der Staay et al., 2006). This impairment in task performance was correlated with the decrease in cholinergic markers. Conversely, studies that selectively removed the septo-hippocampal input (by neurotoxic or immunotoxic abolition) could not support these data. Selective removal did not appear to impair spatial learning and memory (Bizon et al., 2003; Baxter et al., 1995; Niewiadomska et al., 2009), suggesting that deprivation of these cholinergic projections alone is insufficient to induce impairments in hippocampal-dependent spatial learning. Partially supporting either side, a study reported differential effects of immunotoxic lesions. Shen et al. (1996) found that lesioned rats had impaired spatial working memory but not reference memory.

Apart from lesion studies, numerous studies that pharmacologically manipulate cholinergic transmission have been performed to study spatial learning. Inhibition of mAChRs (by muscarinic antagonist scopolamine or atropine) prior to performance of the learning task consistently impaired spatial memory (for review, see Deiana et al., 2011). Effects are generally similar with administration of nAChR antagonists but the effects are weaker than those from administration of mAChR antagonists. Apart from rats, this impairment also extends to humans where administration of scopolamine impaired some aspects of anterograde memory such as acquisition and delayed recall of familiar words and disrupted retention on a distractor task (Beatty, 1986) but did not impair short-term

memory.

Importantly, the effects of ACh antagonists are dependent on the timing of their administration relative to the task onset. Inhibition of cholinergic signalling prior to task onset, and thereby impairing the processes that underlie encoding, impeded learning. However, administration of ACh antagonists after training (i.e. during consolidation) did not induce impairment in recall (Mundy and Iwamoto, 1988; Marighetto et al., 1993). This is consistent with other observations that found varying cholinergic demands at different stages of memory processing: higher during encoding and lower during consolidation (Fadel, 2011). Increased levels of hippocampal ACh release have been observed in spontaneous alternation/working memory tasks (Fadda et al., 1996; McIntyre et al., 2002, 2003), during task acquisition of the radial maze (Stancampiano et al., 1999) and cross-maze (Ragozzino et al., 1999) spatial memory tasks.

Furthermore, microdialysis measurements of hippocampal ACh levels appear to increase as the animal learns. A progressive increase in extracellular ACh during the learning period of a radial-maze task was positively correlated with task performance indicating that levels of ACh map the time course of spatial learning (Fadda et al., 2000). This ACh increase, induced by acquisition, can last for at least 15 min after the task has finished in the hippocampus and cortex. ACh levels in the prefrontal and parital persisted for even longer.

All of these data together suggest that high levels of ACh, promoting the theta rhythm, during learning set circuit dynamics for attention and encoding and thereby facilitate acquisition of spatial information. On the other hand, consolidation of said memory would instead be facilitated by a decrease in hippocampal cholinergic activity that promotes LTP and SWRs (Marighetto et al., 1993; Hasselmo and Mcgaughy, 2004).

1.6 Dopamine: Roles in memory and reward

DA in the brain is a key neurotransmitter for a variety of processes from reward-motivated behaviour, spatial learning and synaptic plasticity to the deficits underlying psychiatric and neurodegenerative disorders such as schizophrenia and PD (Gasbarri et al., 1996; Adcock et al., 2006; Grace et al., 2007; Furth et al., 2013; Brzosko et al., 2015; Schultz, 2016).

The following sections will discuss the effects of dopaminergic modulation on hippocampal learning and memory processes. While there is growing evidence demonstrating the involvement of DA in hippocampal-dependent learning and memory processes, there are very few studies that directly examine the effects of dopaminergic activation on reward learning, specifically when rewards are non-stationary.

1.6.1 Dopaminergic neurons and dopamine receptors

Eleven discrete groups of dopaminergic cell groups, defined as collections of neurons in the CNS that express dopamine-producing enzyme tyrosine hyroxylase (TH) and release DA, have been identified in the brain (Felten and Sladek, 1983; Wallace et al., 1992; German and Manaye, 1993). Given this thesis's focus on hippocampal learning and memory processes, I am particularly interested in sources of DA for the hippocampus. The midbrain provides the main source of DA in the CNS, constituting 90% of the total number of brain dopaminergic cells. The mesolimbic pathway, which is a collection of dopaminergic neurons originating in the VTA (or the A10 cell group in the rat nomenclature) of the midbrain that projects to forebrain limbic regions including the nucleus accumbens, hippocampus and amygdala (Gasbarri et al., 1994), has long been assumed to provide the primary source of dopaminergic input into the hippocampus and is thus classically associated with reward-related learning and working memory. Activation of dopaminergic VTA fibres during

learning enhanced memory and dysfunction of DA neurons in this pathway is associated with psychopathological conditions such as drug addiction and schizophrenia (Rolls et al., 2008).

However, given that VTA DA inputs innervate mostly the ventral hippocampus, recent studies have argued that it may in fact be DA release from noradrenergic fibres originating from the locus coeruleus (LC) that underpin memory associated DA signalling in the hippocampus (Smith and Greene, 2012; McNamara and Dupret, 2017). Optogenetic activation of noradrenergic fibres from the LC (LC-TH$^+$) in hippocampal slice preparations boosted CA3-CA1 synaptic plasticity that was consistent with DA release from noradrenergic terminals (Takeuchi et al., 2016). This finding was further supported in behaviour when burst optogenetic activation of the LC after encoding prolonged persistence of reward location memory (Takeuchi et al., 2016). Moreover, optogenetic activation of LC-TH$^+$ axons significantly increase concentrations of noradrenaline and dopamine concentrations in hippocampal slice preparations and enhanced learning on a spatial object recognition task and the Barnes Maze (Kempadoo et al., 2016).

DA neurons from the retrorubral area (A8) and substantia nigra pars compacta (A9) have also been found to project to the hippocampus (Gasbarri et al., 1996, 1997). The hippocampus also recurrently affects the dopamine system, forming a putative hippocampal-VTA functional loop (Lisman and Grace, 2005; Otmakhova et al., 2013). Nevertheless, accumulating evidence has demonstrated the involvement of VTA DA neurons in hippocampus-dependent learning and memory processes.

At the cellular level, DA's action is mediated by a class of G protein-coupled receptors called DA receptors. There are at least five subtypes: D_1, D_2, D_3, D_4 and D_5 (Kebabian and Calne, 1979; Niznik and Van Tol, 1992; Sibley and Monsma, 1992; Jaber et al., 1996). D_1 and D_5 receptors (D_1-class dopamine

receptors) are coupled to the G protein G_s which activates adenylyl cyclase (AC) that in turn increases intracellular concentration of cyclic adenosine monophosphate (cAMP; Tiberi et al., 1991; Wang et al., 2001). On the other hand, the other three receptor types (D_2-class dopamine receptors) are coupled to the G_i protein which inhibits the production of cAMP via the inhibition of AC (Andersen et al., 1990; Sokoloff et al., 1992; Neve et al., 2004). In addition to their primary action on cAMP signalling, DA receptors have also been found to act through other signalling mechanisms involving alternative G protein coupling, G protein-independent mechanisms, or via ghrelin receptor co-activation (Lacey et al., 1987; Cantrell et al., 1997; Neve et al., 2004; Wellman et al., 2005; Jiang et al., 2006; Beaulieu and Gainetdinov, 2011; Kern et al., 2015). Consequently, DA can have diverse effects on different voltage-gated and calcium-dependent channels which then has wider implications such as influencing the backpropagation of spikes and thereby modulating the induction and expression of synaptic plasticity *in vitro* and *in vivo* (Hoffman and Johnston, 1999; Edelmann and Lessmann, 2011).

1.6.2 Dopaminergic signalling related to reward-related learning

Learning, changes in an animal's behaviour, due to rewards or punishments (consequences) is called *operant conditioning* (Skinner, 1938, 1948, 1951; Ferster and Skinner, 1957). A positive or rewarding event causes behaviour to occur more frequently (*reinforcement learning*) while a negative or punishing event causes behaviour to occur less often (*punishment learning*). A problem that animals face during trial-and-error learning is that they have to associate their actions and decisions with their typically temporally distant consequences (rewards or punishments). How a neural network distinguishes between which past network activities lead to reward and which are irrelevant in the face of this temporal separation is referred to as the *credit assignment problem* (Minsky et al., 1963; Barto et al., 1983; Izhikevich, 2007; Friedrich et al., 2011). DA,

with its importance in reinforcement learning and reward-motivated behaviour, is a strong candidate for a brain mechanism responsible for solving this problem.

Reward processing was first studied in the 1950s by Olds and Milner (Olds and Milner, 1954) when they implanted electrodes into the brains of rats and stimulated the cells surrounding the tip of the electrode with electric current whenever the rats pressed a lever. When the electrode was placed in structures connected with DA neurons, the rats pressed the lever again and again, to get more electric shocks to their brains. Repeated experiments in cats (Wilkinson and Peele, 1963) and monkeys (Bursten and Delgado, 1958) yielded similar results, demonstrating that activation of DA neurons induce learning and approach behaviour, linking brain function in a causal way to behaviour.

Moreover, DA neurons are not only activated when reward is encountered, simply presenting a subject money, food, or a stimulus, such as light, picture or sound that predicts a reward also make the majority of DA neurons active (Adcock et al., 2006; Cohen et al., 2012). The greater the reward, the stronger the DA response. These reward-predicting stimuli are called conditioned rewards and DA neurons treat these stimuli and real rewards the same way (Schultz, 2016). Furthermore, these predictive stimuli meant animals can plan ahead and make informed decisions. Therefore, by signalling both rewards and reward-predicting stimuli, DA neurons can facilitate learning and decision-making by providing information about past and future rewards.

Interestingly, if the received reward matches their expectation, this DA response disappears. Phasic changes in DA activity are strongly linked to reward prediction error signalling, that is, the degree to which the received reward is different from predicted (Chang et al., 2015). Indeed, single-unit recordings show that dopaminergic neurons in the midbrain increases firing with positive prediction errors (reward received was greater than expected) and firing was briefly depressed with negative prediction errors (reward received was less than

expected; Schultz et al., 1993, 1997; Schultz, 1998; Pan et al., 2005; Zaghloul et al., 2009; Cohen et al., 2012). Interestingly, DA neurons do not respond if reward received is equal to expected. These DA responses have been found in monkeys, humans and rodents and also verified by computational models. They highlight the importance of reward prediction errors in driving reward learning. When a prediction error exists, the animal updates its prediction and changes future behaviour, the animal is learning. When there is no prediction error, prediction and behaviour remain unchanged, nothing is learnt.

Furthermore, activity of DA neurons is not only phasic, but can also be tonic (Schultz and Dickinson, 2000; Schultz, 2002; Howe et al., 2013). DA signals gradually increase as the animals navigate through mazes to reach remote rewards (Howe et al., 2013). This ramping of DA response scale flexibly with distance and size of rewards, and as such provide a continuous estimate of the proximity of rewards and can evaluate the relative values of rewards when more than one reward option are available. Therefore while phasic dopamine is important as a teaching signal for reward learning, tonic dopamine is suggested to be important for motivational drive (Schultz, 2002, 2007; Howe et al., 2013; Schultz, 2016). Recent studies also suggest other heterogeneous DA functions such as value-coding versus salience-coding (Matsumoto and Hikosaka, 2009) or update-type versus sustain-type (Kim, Ghazizadeh and Hikosaka, 2015).

1.6.2.1 Dopaminergic modulation of hippocampal spatial learning at cellular networks

The interactions between DA and neural network activity is poorly understood despite both of their importance in cognitive function (Whittington et al., 2011; Gandal et al., 2012). Nevertheless, a few studies have pointed to an important role in DA in modulating network dynamics. EEG studies in healthy human subjects found haloperidol, a first generation antipsychotic, suppressed gamma power and this effect was attributed to inhibition of D_2 receptors (Ahveninen et al., 2000). Genetic mouse models such as dopamine-transporter knockout

(DAT1-ko) mice, which systematically induce hyperdopaminergia, showed elevated gamma power in the hippocampus (Demiralp et al., 2007). Meanwhile, *in vitro* electrophysiological studies of electrically or pharmacologically induced gamma oscillations in rodent hippocampal slices yielded conflicting results. Application of DA increased the power and duration of stimulation-induced gamma oscillations (Wójtowicz et al., 2009) but decreased the duration of CA1 carbachol-induced gamma oscillations (Weiss et al., 2003) and CA1 or CA3 kainate-induced gamma oscillations (Wójtowicz et al., 2009; Jones et al., 2012). The different mechanisms underlying these various types of gamma oscillations may underlie these variations.

Pharmacological analysis found that direct stimulation of D_3 receptors by the selective agonist PD128907 inhibited hippocampal gamma oscillations (Schulz et al., 2012). This finding is supported by a recent study showing that the decreased power, coherence and dynamics of gamma oscillations by activation of D_3 receptors is accompanied by reduced CA3 pyramidal cell activity and reduced precision of spike phase coupling to the gamma cycle (Lemercier et al., 2016). Moreover, other DA receptors have been implicated in gamma oscillations. Clozapine, an atypical antipsychotic with moderate selectivity for D_4 and 5-HT_3 receptors, and haloperidol, a typical antipsychotic with D_2 antagonistic properties, reduced the amplitude of ACh-induced gamma in hippocampal slice preparations (Schulz et al., 2012). Another study found that activation of D_4 receptors by the selective agonist PD168077 enhanced gamma oscillations in the CA3 (Andersson et al., 2012). Lastly, activation of D_1 receptors decreased the power of carbachol-induced gamma oscillations (Weiss et al., 2003). Taken together, these data reveal that the effects of DA on network oscillations are complex and most likely depend on a combination of factors such as mechanisms of gamma oscillation induction and DA receptor pharmacology.

DA has also been implicated in hippocampal theta oscillations (Xu et al., 2016; Nakagawa et al., 2000). While depletion of DA in the hippocampus decreases

theta activity (Nakagawa et al., 2000), injection of DA or non selective DA agonist apomorphine into the MS and diagonal band increased hippocampal theta activity in anaesthetised rats. This enhanced effect can also be blocked with treatment of haloperidol (Miura et al., 1987). Furthermore, direct infusion of DA into the prefrontal cortex (PFC) of anaesthetised mice enhanced hippocampal-prefrontal theta coherence (Benchenane et al., 2010), indicating another mechanism by which DA could modulate reward-related learning by tagging tightly synchronised reward-predictive cell assemblies for subsequent stabilisation by STDP.

DA is a particularly important neuromodulator of synaptic plasticity of the hippocampal place cell network, a prime cellular model for the encoding of spatial memories. It is well established that DA profoundly influences STDP (Thivierge et al., 2007; Florian, 2007; Izhikevich, 2007; Zhang et al., 2009; Fremaux et al., 2010), e.g. application of DA can increase the time window for t-LTP induction (Ruan et al., 2014; Yang and Dani, 2014) and rescued t-LTP in sucrose-induced STDP deficit (Edelmann and Lessmann, 2011). As described earlier (section 1.5.2.1), STDP can be further modulated after its induction by subsequent neuronal activity and neuromodulatory input. Not only can application of DA before and during the post-before-pre induction protocol that normally results in t-LTD cause expression of t-LTP, application of DA immediately after the t-LTD induction protocol can retroactively convert t-LTD to t-LTP (Brzosko et al., 2015). This highlights the power of DA as a positive reinforcement signal and has important implications for reverse replay.

The reactivation of previously encoded place cell sequence during SWR events (forward or reverse replay) is implicated in hippocampal memory consolidation as replay could allow the evaluation of event sequences that lead to reward. The path that preceded reward discovery is valuable and locally initiated reverse replay has been proposed as a mechanism for evaluation of previously visited environments that led to its present rewarded location and retroactively

assign value to its path (Davidson et al., 2009; Ambrose et al., 2016). DA signalling, in particular, is of special interest in facilitating this value assignment as pairing the reverse replay with a slowly decaying DA signal could provide a value gradient for subsequent reward-oriented behaviour. Indeed, hippocampal replay events have been shown to be promoted by reward and activation of DA neurons in the midbrain (McNamara et al., 2014; Gomperts et al., 2015).

1.6.2.2 Dopaminergic modulation of hippocampal spatial learning in behaviour

The importance of DA in hippocampal spatial learning have been demonstrated in multiple recent behavioural experiments employing genetic mouse models, pharmacological and surgical treatments. For example, D_1 (but not D_3) receptor knockout mice display spatial learning deficit (El-Ghundi et al., 1999; Tran et al., 2008), intra-hippocampal administration of D_1-like receptor antagonist during encoding similarly impairs long-term spatial memory formation (da Silva et al., 2012) and the persistence of spatial memory in a one trial delayed matching-to-place task (O'Carroll et al., 2006). Infusion of D_1 agonist post-training improves retention and enhances the spontaneous recovery of the original spatial preference after reversal learning. Notably, non-spatial memory is not affected (da Silva et al., 2012; Clausen et al., 2011).

A number of lesion studies have also pointed to an important function of the DA mesolimbic pathway. Loss of mesencephalic dopaminergic neurons impaired performance in a spatial version of the Morris water maze, but not in the inhibitory avoidance and the cued version of the Morris water maze task (Gasbarri et al., 1996). Likewise, another study found that lesioning of dopaminergic neurons in the VTA led to impaired performance in a reference memory task while execution of working memory tasks appeared to be unaffected (Wisman et al., 2008). Further analysis of the search paths of DA-depleted animals showed that these animals adapt a search strategy on a given testing day but

they fail to recall this strategy on the following day, suggesting an impairment in information storage and/or recall.

Similarly, the importance of midbrain DA neurons in modulating hippocampus-dependent episodic memory, including spatial memory, has also been demonstrated in humans in a number of recent neuroimaging studies (Wittmann et al., 2005; Schott et al., 2006; Shohamy and Adcock, 2010). First, activation of dopaminergic inputs from the midbrain following reward cues enhances hippocampus-dependent long-term spatial memory formation (Wittmann et al., 2005; Wolosin et al., 2012; Marsh et al., 2010). Second, brain activation in the midbrain and the hippocampus preceding reward stimulus can predict subsequent declarative memory formation, suggesting that reward motivation facilitates memory formation via DA release in the hippocampus prior to learning (Adcock et al., 2006). Interestingly, the information about reward value may be directly encoded in reward-motivated episodic memories (Kuhl et al., 2010). Third, midbrain and hippocampus have been postulated to facilitate generalisation of information by enhancing dynamic integration of individual experience episodes. Participants who showed robust activation of both midbrain and hippocampus during learning were more likely to later successfully generalise what they learned to solve novel problems (Shohamy and Wagner, 2008). This integrative encoding could enable animals to link multiple past memories to guide choices in new situations.

Whilst together, these neuroimaging, pharmacological and lesion studies have pointed to the importance of DA modulation on hippocampal spatial memory, they have yet to demonstrate a causal relationship between the mesolimbic pathway and hippocampal memory systems. Recent optogenetic tools, which allow temporally precise activation or inactivation of specific neuronal groups, have been used to selectively stimulate VTA DA neurons in rodents and monkeys (Tsai et al., 2009; Witten et al., 2011; Chang et al., 2015; Steinberg et al., 2013; Stauffer et al., 2016). Their results demonstrated that phasic activation of

midbrain DA neurons can drive behavioural conditioning and augments the choice preferences for the option associated with DA stimulation. However, whether transient DA signal alone can drive reward learning in a hippocampus-dependent spatial memory task and its effects on reversal learning remain uncertain.

1.7 Aim of this thesis

Although it is well established that acetylcholine and dopamine modulate hippocampal learning and memory processes, much of our knowledge to date has been derived from exogenous application of receptor agonists or blockers. This technique lacks temporal precision and reveals few clues to the context of neurotransmitter action. Optogenetics provides a promising *in vitro* and *in vivo* method to study the architecture and function of neural circuits with high spatial and temporal precision. Neurons may be controlled with expression of channelrhodopsins (conduct cations and depolarise neurons upon illumination) or archaerhodopsins (transport protons and hyperpolarise neurons upon illumination) that will quickly and selectively activate or inhibit particular cell types, synapses or neuronal pathways in specific regions of the brain (Boyden et al., 2005; Han et al., 2011).

Using this technique, I aim to directly examine the impacts of cholinergic activation and inactivation and dopaminergic activation on hippocampal network oscillations, before translating these findings to investigate whether there are any manifestations of cholinergic and dopaminergic modulation at the behavioural level. Given the possible biphasic action of acetylcholine and dopamine, it may be helpful to explore whether these neuromodulators differentially affects memory encoding and consolidation in various short-term and long-term memory spatial memory tasks.

Specifically, the objectives of this thesis are:

1. to evaluate the effects of (i) cholinergic activation, (ii) cholinergic inactivation and (iii) dopaminergic activation on hippocampal local field potentials and ensure that light stimulation on the optogenetic mouse models used for behavioural experiments have physiological effects

2. to investigate the effects of cholinergic activation and inactivation on spatial short-term memory and compare their effects at different phases of a couple of short-term memory tasks

3. to establish the effects of cholinergic activation on different phases of place learning during a long-term spatial memory task

4. to examine the effects of (i) dopaminergic activation and (ii) cholinergic inactivation on spatial place learning and reversal learning.

Chapter 2

Materials and Methods

2.1 Mice

Animal care and experimental procedures were conducted in accordance with U.K. Home Office regulations under the Animals (Scientific Procedures) Act of 1986 under appropriate personal and project licences. Mice were housed in polycarbonate cages of 2-10 animals and had access to food and water ad libitum, except when on food restriction during behavioural testing. Holding facilities were maintained at approximately 22 °C, 60-70% humidity, and with a 12-hour light-dark cycle (7 a.m. to 7 p.m.).

Three types of transgenic mice were used for experiments presented in this thesis.

1. *ChAT-Ai32* mice which are offspring of the ChAT-Cre line (Jackson Laboratories, stock #006410), mice that express the Cre recombinase under the control of the choline acetyltransferase (ChAT) promoter (Rossi et al., 2011), crossed with the Cre-reporter Ai32 line (Jackson Laboratories, stock #012569) bearing a Cre-dependent, enhanced YFP (eYFP)-tagged channelrhodopsin-2 (ChR2)-containing expression cassette (Madisen et al., 2012). ChAT-Ai32 mice express ChR2-eYFP proteins in all cholinergic cells and activate upon illumination by 473 nm light.

2. *ChAT-Ai40D* mice which are offspring of the ChAT-Cre line crossed with the Ai40D line (Jackson Laboratories, stock #021188) bearing a Cre-dependent, enhanced GFP (eGFP)-tagged Archaerhodopsin-3 (ArchT) fusion protein (Maksimovic et al., 2014; Gizowski et al., 2016). ChAT-Ai40D mice express ArchT in all cholinergic cells and activate upon illumination by 561 nm light.

3. *DAT-Ai32* mice which are offspring of the dopamine transporter::internal ribosome entry site-Cre (DAT::IRES-Cre mice; Jackson Laboratories, stock #006660) line expressing Cre recombinase activity directed to dopaminergic neurons (Zhuang et al., 2005; Bäckman et al., 2006) crossed with

the Cre-reporter Ai32 line bearing a Cre-dependent, eYFP-tagged ChR2-containing expression cassette. DAT-Ai32 mice express ChR2-eYFP proteins in dopaminergic cell groups (substantia nigra, ventral tegmental area (VTA), and the retrorubral field) and activate upon illumination by 473 nm light.

Control mice consisted of transgenic negative littermates or wild-type (WT) C57BL/6J mice bred in-house or bought from Jackson Laboratories. Only male mice were used for *in vivo* behavioural testing.

2.2 Optogenetic stimulation

2.2.1 Channelrhodopsin

ChR2 was excited using a blue-light 473 nm diode-pumped solid-state laser (Ciel, Laser Quantum, Cheshire, UK) that was assembled on a breadboard set-up (optical components from Thorlabs and Doric Lenses) and collimated into an aperture-matched fibre-optic patch cord (Doric Lenses). Custom-made stimulation protocols executed in Igor Pro (Wavemetrics, Oregon, USA) were used to generate 50 ms-long square pulses at 10 Hz (apart from data for Figure 3.7 where the pulses were varied at the specified frequency), controlled with a low vibration shutter combined with a shutter controller (Stanford Research Systems, Sunnyvale, U.S.A.).

For anaesthetised animal recordings, the fibre-optic patch cord was stripped at one end to illuminate neurons in the MS or VTA. The light output was adjusted to 5-30 mW at the fibre tip using a thermosensor (Thorlabs).

For freely moving animal recordings and behavioural testing, a cannula containing a fibre optic was implanted into the desired site (see section 2.3.2 and

2.4) and a patch cord was used to connect the laser to the cannula via a cubic zironia sleeve (Figure 2.2D). The light output was adjusted to 25±1 mW at the zironia sleeve-end of the patch cord. In order to allow the mouse free rotation and equal movement in all directions, a rotary joint (Doric Lenses) attached to the ceiling in the centre of the behavioural testing apparatus was used to connect the patch cord attached to the laser and the patch cord connected to the implant. All the equipment were placed from a sufficient height to ensure that the patch cord would never constrain the mouse's movement while in the behavioural apparatus.

2.2.2 Archaerhodopsin

ArchT was excited using a yellow laser-light from a solid-state laser diode (561 nm; Laser 2000), set up similarly to the blue laser. However, rather than 50-ms long pulses, ArchT was excited continuously for the duration of the stimulation. The light output was adjusted to 26±1 mW at the fibre tip.

2.3 *In vivo* electrophysiology

2.3.1 Recordings in anaesthetised animals

Mice were anaesthetised with intraperitoneal injections of 1.2 gkg^{-1} urethane (Sigma-Aldrich, Missouri, USA) and their head was fixed in a stereotaxic frame (Kopf Instruments, Tujunga, USA). A heating pad was used to help maintain body temperature at 35±1 °C. The head was shaved, levelled and a craniotomy was made above the MS or VTA and hippocampus.

Simultaneous optical activation in the MS (AP: +1 mm, ML: 0 mm, DV: –3.6 mm, coordinates from Bregma) or VTA (AP: –3.1 mm, ML: ±0.5 mm, DV:

–4.3 mm) with a stripped optical fibre (200 μm, 0.22 NA; Doric Lenses) and electrical recordings in the hippocampus (ML: +2.4 mm, AP: –2.46 mm, DV: –2.5 mm) using an extracellular parylene-C insulated tungsten microelectrode (127μm diameter, 1 MΩ; A-M Systems, Hinckley, UK) were performed (Figure 2.1). Multi-unit activity (MUA) in the MS or VTA were recorded using an extracellular tungsten microelectrode (127μm diameter, 1 MΩ; A-M Systems; Figure 2.1A, B). During the experiment, the surface of the exposed skull was covered with saline (0.9% NaCl).

Surgery was terminal and at the end of the experiment, a 3 mA current was passed through the recording electrode for 2 s, causing a lesion at the recording site. Coronal slices of the hippocampus and the MS or VTA were prepared using a vibratome (VT1200S, Leica Biosystems, Milton Keynes, UK) and visualised under a bright-field microscope to verify the recording site.

2.3.1.1 Multi-unit activity recordings

Multi-unit activity (MUA) was recorded in the MS or VTA using a 2-channel microelectrode amplifier (1800 Microelectrode AC Amplifier, A-M Systems) at an acquisition rate of 20 kHz and acquired using an ITC18 acquisition data board (Instrutech, Colorado, USA) and custom procedures in IgorPro (Wavemetrics, Oregon, USA).

A baseline 15-30 seconds of MUA was first recorded. This was followed by 30-50-ms long pulses of 473 nm light at 10 Hz at 25 mW to stimulate ChR2-containing cells or 30-60-s long continuous illumination of 25 mW 561 nm light to stimulate ArchT-containing neurons to test the effects of light illumination. MUA data was analysed in MATLAB using custom protocols. Data were first digitally filtered using finite impulse response (FIR) filters between 300-3000 Hz to keep spike activity and preserve linear phase. Data were then imported

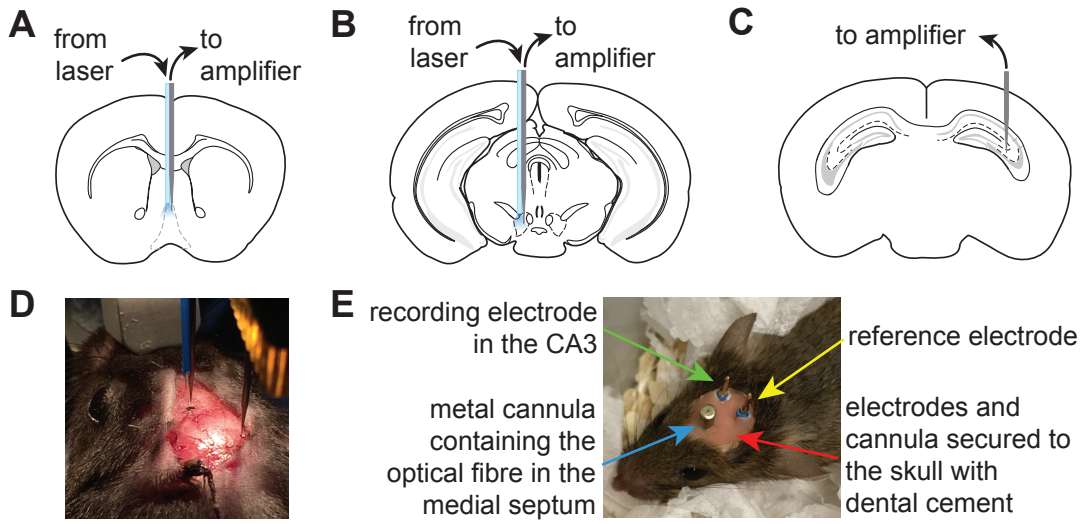


Figure 2.1: In *vivo* electrophysiology. (A) Schematic of an optical fibre and an extracellular microelectrode lowered into the MS of ChAT-Ai32 or ChAT-Ai40D mice for light delivery to stimulate ChR2/ArchT-expressing neurons and to record neuronal spiking activity. **(B)** Schematic of an optical fibre and an extracellular microelectrode lowered into the VTA of DAT-Ai32 mice to activate ChR2-expressing neurons and record multi-unit activity. **(C)** Schematic of an extracellular microelectrode lowered into the CA3 of the hippocampus to record local field potentials and any possible response from light stimulation. **(D)** In an anaesthetised animal recording, the optrode was lowered through a craniotomy into the brain until the extracellular microelectrode tip was in the CA3. **(E)** An adult ChAT-Ai32 mouse after the implantation for freely-moving recordings showing the connecting pins for the CA3 recording electrode (green arrow), reference electrode (yellow arrow) and the metal cannula containing the optical fibre (blue arrow), which were cemented to the skull with dental cement (red arrow).

into MATLAB and individual spikes were counted using a voltage threshold set to:

$$Thr = 5 \times \sigma; \qquad \sigma = median\{\frac{|x|}{0.6745}\}$$

where x is the bandpass-filtered trace and σ is an estimate of the standard deviation of the background noise (Donoho and Johnstone, 1994).

For VTA recordings, the traces were put into Wave_clus by Quiroga et al. (2004) for unsupervised spike clustering. Briefly, after the spikes were detected, distinctive features of the spikes' shapes were extracted using wavelet transform which optimally decompose the signal in both the time and the frequency

domains. These features (i.e. a selected set of wavelet coefficients) were then used to classify the spikes with superparamagnetic clustering (SPC). SPC is a method implemented as a Monte Carlo iteration of a Potts model, allowing the automatic classification of the data without assumptions such as low variance or Gaussian distributions (Quiroga et al., 2004).

Statistical analyses were performed using SPSS or Prism GraphPad and significance was assessed by unpaired two-tailed *t*-test. All values were given as mean±S.E.M., and numbers (n) refer to the number of recordings, obtained from at least two animals.

2.3.1.2 Local field potentials recordings

Local field potentials (LFP) in the hippocampus were recorded when the mouse was in slow wave (SW) as well as rapid eye movement (REM; initiated by continuous tail pinch) sleep and acquired using the same procedures as above.

Similar to MUA recordings, a baseline of 15-30 seconds was first recorded, followed by brief (30-50 ms) pulses of 473 nm light at various frequency (2-20 Hz) and light intensity (5-30 mW) for 30-60 seconds to stimulate ChR2-containing neurons in ChAT-Ai32 or DAT-Ai32 mice or 30-60-s long continuous 561 nm light illumination in ChAT-Ai40D mice.

After at least one set of control recordings, some of ChAT-Ai32 mice received an intraperitoneal injection of 2 $mgkg^{-1}$ scopolamine (Tocris, Bristol, UK), a muscarinic cholinergic receptor antagonist (Drevets et al., 2013). Scopolamine hydrochloride was prepared fresh daily in saline and was administered as a volume of 1 mL/kg.

LFP data was analysed in Igor Pro (Wavemetrics) using custom protocols. The power and frequency of the LFP were calculated using Welch's power spectral density (PSD) which helps reduce noise in the power spectrum by reducing the frequency resolution (Musson and Li, 2010). The power of slow oscillations (0.5-2 Hz), theta oscillations (2-6 Hz) and slow gamma oscillations (20-40 Hz) were estimated as the area under the PSD of the frequencies in question. As the power of these frequencies vary from recording to recording and since I was most interested in the effects of light stimulation on power, the change in power during light stimulation in relation to the power during baseline was calculated. The normalised area under the PSD curve was calculated as:

$$Normalised\ ratio = \frac{Area\ under\ PSD\ curve(During\ stimulation)}{Area\ under\ PSD\ curve(Before\ stimulation)}$$

To correlate stimulation events and theta oscillations recorded in the LFP, a continuous wavelet transform (CWT) was calculated using a normalised Morlet wavelet transform for each trace and a spectrogram of the LFP signals was produced.

Statistical analyses were performed using SPSS or Prism GraphPad and significance was assessed by unpaired two-tailed *t*-test or by one-way ANOVA with a between subjects factor of treatment (with three levels: no light, 30 s light stimulation and 30 s light stimulation + scopolamine) or by one-sample *t*-test. If significant differences were found, further post-hoc analysis was performed using the Tukey test (when sample sizes were equal) or the Gabriel's procedure (if sample sizes were slightly different as it has greater power; Field, 2013). All values were given as mean ± S.E.M., and numbers (n) refer to the number of recordings, obtained from at least two animals.

2.3.2 Recordings in freely-moving animals

ChAT-Ai32 mice (8 weeks old) were anaesthetised with 2-4% isoflurane at 0.6-1.5 Lmin^{-1}). Their head was fixed in a stereotaxic frame (Kopf Instruments) and body placed on a heating pad to help maintain body temperature of 34±1 °C. The head was shaved, skin was incised and the skull was exposed. The head was levelled and three small holes (<0.5 mm in diameter) were made through the skull. A 14 mm-long platinum-iridium extracellular microelectrode (127 μm diameter, 1 MΩ; Microprobes, Maryland, US) was lowered into the right CA3 (ML: +2.4 mm, AP: –2.46 mm, DV: –2.5 mm) and a 12 mm-long platinum-iridium reference microelectrode (127 μm diameter, 1 MΩ; Microprobes) was placed in the contralateral cortex (ML: –1.8 mm; AP: –3.8 mm; DV: –0.5 mm). A mono fibre-optic cannula (4-mm long, 200 μm diameter, 0.22 Numerical Aperture, NA; Doric Lenses, Quebec, Canada) was lowered into the MS (AP: 1 mm, ML: 0 mm, DV: –3.55 mm). These electrodes and optical fibre were secured to the skull using dental cement (CB Metabond; Prestige Dental, Bradford West, U.K. and Simplex; Claudius Ash, Stevenage, U.K.; Figure 2.1E). The scalp incision was sutured, and to aid recovery, anti-inflammatory and analgesic drugs (2 mgkg^{-1} meloxicam; 0.1 mgkg^{-1} buprenorphine) were administered subcutaneously.

After at least 3 weeks of recovery, mice were placed in a clear rectangular box (5 $\times$30$\times$10 cm high). A white food well (1.5 cm high) containing food reward (sweetened condensed milk) was placed in the centre of the box for the mice to mimic consummatory behaviour during an appetitive behavioural task. Local field potentials (LFP) were recorded in the hippocampus using the same procedures as described in section 2.3.1.2.

2.4 Implant surgery for behavioural experiments

Mice (>4 weeks old) were anaesthetised with 2-4% isoflurane at 0.6-1.5 $Lmin^{-1}$), their head fixed in a stereotaxic frame (Kopf Instruments) and body placed on a heating pad to help maintain body temperature to 34-36 °C. The head was levelled and a craniotomy was performed. For ChAT-Ai32 or ChAT-Ai40D animals and their WT control mice, a mono fibre-optic cannula (4 mm-long, 200 μm diameter, 0.22 NA; Doric Lenses) was positioned above the MS (AP: +1 mm, ML: 0 mm; DV: –3.55 mm; Figure 2.2A). For DAT-Ai32 mice and their WT control mice, a dual fibre-optic cannula (4.2 mm-long, 1 mm distance between fibre tips, 0.22 NA; Doric Lenses) was placed just above the VTA (AP: –3.1 mm, ML: $\pm$10.5 mm, DV: –4 mm; Figure 2.2B).

The cannula was secured to the skull using dental cement (CB Metabond; Prestige Dental and Simplex; Claudius Ash; Figure 2.2C). The scalp incision was sutured, and to aid recovery, anti-inflammatory and analgesic drugs (2 $mgkg^{-1}$ meloxicam; 0.1 $mgkg^{-1}$ buprenorphine) were administered subcutaneously. Mice were returned to their home cages and left for at least 2 weeks, or until they were fully recovered, before behavioural testing.

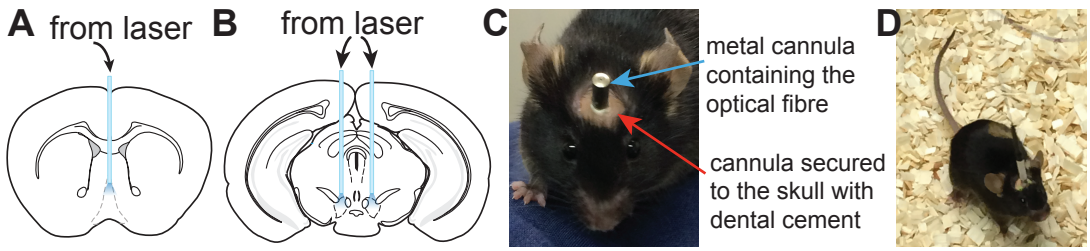


Figure 2.2: Implant surgery and light delivery for behavioural experiments. Placement of the optical fibre in the **(A)** MS and **(B)** the VTA. **(C)** A 0.22 NA optical fibre was contained in a metal cannula (50 mm by 7.5 mm; blue arrow), which was cemented to the skull with dental cement (red arrow). **(D)** Mice were securely connected to a fibre-optic patch cord coupled to the laser light via a cubic zirconia sleeve during behavioural testing.

2.5 Behavioural experiments

Mice began behavioural testing on average 4 weeks after surgery to allow time for surgery recovery and handling. Prior to the start of behavioural testing, each mouse was handled for two weeks to habituate them to the experimenter and familiarise them to the connection of the implant.

2.5.1 Spontaneous alternation T-maze task

The spontaneous alternation T-maze task is a spatial learning and memory task used to assess the hippocampal-dependent short-term memory of mice. It is based on the rodents' strong preference to explore a novel and different environment (Deacon and Rawlins, 2006).

The T-maze was made up of a central arm and two choice arms. The arms were all enclosed by 30 cm by 10 cm, 20 cm high black acrylic walls and a white floor. The maze was placed in a fixed position in the testing room such that shading on both choice arms was similar. Before the start of every trial, a removable 5 cm-long divider was placed in between the two choice arms so that the mice would only receive sensory input from their chosen arm while they were deciding which arm to enter. The mice started in a pseudorandom order, which varied across trials. Just prior to placing the mice in the maze, their fibre-optic implant was connected to the laser and illumination started for any 'light on' phases and 10 Hz stimulation continued for the duration of the phase. For any 'light off' phases, the mice were still connected to the laser but did not receive any stimulation.

Mice were first individually placed at the start of the central arm, facing outwards. They were allowed to enter either the left or right choice arms (Phase 1). As soon as all 4 paws crossed the opening of a choice arm, a black barrier was placed to contain it in its chosen arm. After a 30 s-long exploratory

period, the mouse, divider and barrier were removed from the maze. The mouse was placed back into its home cage for the duration of the delay period (except for when the delay period was 0 s). Once the delay period had elapsed, the mouse was placed back into the start of the central arm and their subsequent arm choice was recorded (Phase 2). If the mouse chose the novel arm, this was counted as a spontaneous alternation (Figure 2.3A). Mice tend to enter the arm not visited before, indicating memory of the previous choice (Deacon and Rawlins, 2006). Mice were tested, at most, twice per day. A trial was aborted if the mouse took >30 s to make an arm choice as delay times could affect their performance (Zhang et al., 2013).

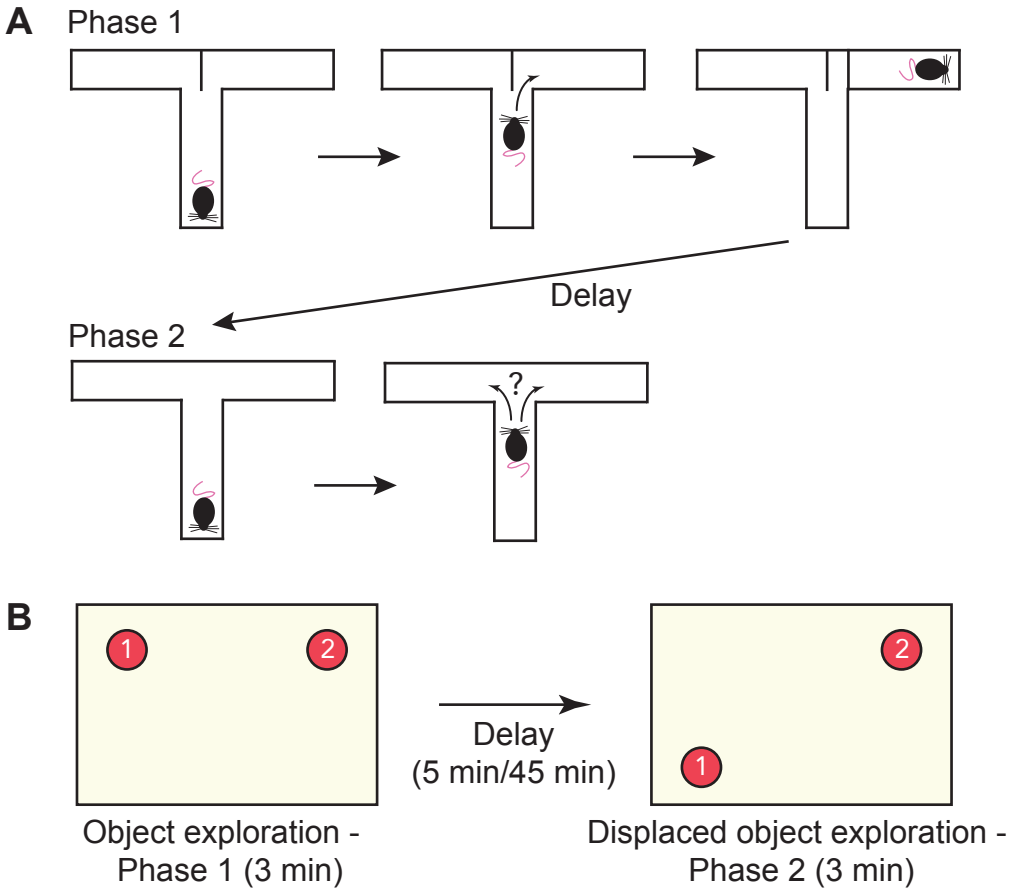


Figure 2.3: Schematic of behavioural tasks used to test short-term memory. **(A)** The spontaneous alternation T maze task used to test short term spatial memory. **(B)** The spontaneous location recognition task used to test short term object location memory.

2.5.2 Spontaneous Location Recognition Task

The spontaneous location recognition (SLR) task (previously described by Ennaceur et al., 1997) is designed to assess the mouse's ability to discriminate whether an object it had previously encountered in the same test arena had changed location. Rodents naturally spontaneously spend more time exploring the object in the novel location compared to the object in its previous location.

Testing took place in a black wooden open rectangular box (50×30×20 cm high) with the floor covered with approximately 1 cm thick of bedding. A digital video camera (Sony™) was mounted above the box to record all trials. The mice were presented with novel objects for each trial. The objects were placed close (approximately 10 cm) to two adjacent or opposite corners, which were randomly selected for each mouse and counterbalanced for the different objects and light conditions. The objects were randomly shaped (dimensions approximately 10×4×4 cm) and had not been seen by the mice previously. Sixteen different pairs of objects were used for each trial and were randomly assigned to different mice for various light conditions and delay periods. The objects were secured to the floor of the maze using Blu-tack™. All objects and the arena were wiped with 70% ethanol after each trial. The testing room was lit with white light and had distinct distal and proximal spatial cues.

Prior to the start of the SLR task, each mouse was given 4 consecutive daily sessions of habituation, in which they were allowed to freely explore the empty test area for 6 minutes. This was intended to allow the mice to become familiar with the spatial cues in the room and reduce anxiety.

Each trial consisted of two phases: *sample* phase (Phase 1) and *test* phase (Phase 2; Figure 2.3B). During the Phase 1, the mouse was placed in the centre of the arena and was allowed to explore the arena for 3 mins. It was shown two identical objects placed in adjacent corners of the arena, along the length

of the open box. The mouse was then placed back in their home cage for a delay period (30 s, 5 mins, 10 mins, 30 min or 3 hours). After the delay period, each mouse was returned to the arena for Phase 2 and allowed to explore for 3 mins. In Phase 2, the mouse was presented with the same two identical objects previously used during Phase 1 but one in its previous, familiar location (i.e. non-displaced object) and the other placed in a novel location (i.e. displaced object), in the corner adjacent to the original position such that the two objects were in diagonal corners. All mice were tested concurrently; each mouse received 1-2 trials for each light condition and each delay period, with a minimum interval of 48 h between each trial. The sequencing of the delay periods and light conditions was counterbalanced amongst the cohort. The left-right positions of the displaced and non-displaced objects and objects presented for the four light conditions were also counterbalanced.

Exploration was defined as the mouse directing its nose to the object at a distance of $\leq$2 cm. Climbing, sitting, or chewing on the object was not included as exploratory behaviour. The time spent exploring each object were recorded using JWatcher V1.0, written in Java™ (JWatcher, USA).

2.5.2.1 SLR Data analysis

Repeated measures ANOVA were used to compare the time spent exploring each of the objects in Phase 2 for the various light conditions. It should be noted that this task is based on spontaneous exploratory activity, and as a result, there was the possibility of individual mice having a preference for a specific place that is independent of the familiarity/novelty of the object location. Thus, variance levels can vary markedly from trial to trial.

In addition, the following measures were also calculated and analysed: (i) *e1*, the total time spent in exploring the two identical objects in Phase 1; (ii) *p1*,

the proportion of time spent exploring one of the objects (e.g. Object 1) during Phase 1; (iii) *e2*, the total time spent exploring the two objects in Phase 2; (iv) *d2*, the discrimination ratio, i.e. the difference between the time spent exploring the displaced object (Od) and non-displaced object (On) expressed as a proportion of the total time spent exploring the two objects in Phase 2 (i.e. e2; Table 2.1). d2 is 0 if the mouse spent the same amount of time exploring both objects during Phase 2.

Table 2.1: Index of the different measures involved in the spontaneous location recognition task. *e1* = the total time spent exploring the two identical objects O1 + O2 in Phase 1; *p1* = the proportion of time spent exploring one of the objects (e.g. O1) during Phase 1; *e2* = the total time spent exploring the object in the non-displaced location (On) and displaced object (Od) in Phase 2; *d2* = the discrimination ratio, i.e. difference in time spent exploring the two objects in Phase 2 divided by the total time spent exploring the two objects.

Phase 1		Phase 2	
e1	p1	e2	d2
O1+O2	O1/(O1+O2)	On+Od	(Od–On)/(On+Od)

2.5.3 Appetitive Y-Maze Task

To evaluate long-term spatial memory, I used the appetitive Y-maze task previously described by Shipton et al. (2014). Mice were trained to find the food reward on one of the arms of an elevated Y-maze using extra-maze spatial cues.

The Y-maze was made of three grey painted wooden arms (50×13 cm bordered by 1 cm high white plastic walls), extending from a central triangle. Plastic food wells (1.5 cm high) were positioned 5 cm from the distal end of the arms and secured to the maze with magnets. Grey tape marked 20 cm from the distal end of the arm to indicate the mouse's arm choice. The maze was elevated 82 cm from the floor. The testing room was lit with white light and had distinct painted black and white visual cues made from cardboard and standard furniture. The visual cues were placed such that they did not align

with the end of the arms.

Mice were food-restricted, fed once daily after habituation or testing and allowed to maintain at least 85% of their free-feeding body weight. Mice were introduced to the food reward (0.1 mL of sweetened condensed milk diluted 50:50 with water) in their home cages to overcome neophobia and then pre-trained on the elevated Y-maze in a different room to where behavioural testing would occur. Behavioural testing occurred once their motivation to search for the reward is high (reward reached in less than 20 seconds for three consecutive trials).

Mice began the task facing outwards from the centre of the maze in either the left or right arm relative to the target arm (Figure 2.4A). Each mouse received ten trials per day for ten consecutive days (Day 1-10). On the tenth day, the reward was given after the arm choice was made as a control for mice locating reward by odour (post choice baiting; P.C.B.). Seven days after block 10, the mice were given ten more trials to test whether they remembered the location of the food reward (Day 17). On each day, they had five starts from the left of the target arm and five starts from the right in a pseudorandom order with no more than three consecutive starts from the left or right.

Mice were randomly assigned a rewarded target arm (designated a, b and c according to its fixed position relative to extramaze cues). To test the effects of light stimulation on various phases of the Y-maze task (Figure 5.2), mice were randomly assigned to four groups to test four light conditions (i: no light; ii: light on throughout the maze; iii: light on only until the reward (taped grey line) is reached – navigation; and iv: light on only during reward (from the taped grey line); Figure 2.4B). Target arm assignments were counterbalanced such that at least one mouse of each experimental group were designated to each arm.

Mice were started in a pseudorandom order within the cage that varied across trials and the inter-trial interval (ITI) was approximately 11-12 minutes. Before being placed on the maze, the implant was connected to the laser and depending on their experimental group, 50 ms-long pulses of 473 nm light (25 mW at a frequency of 10 Hz) were applied to the MS. An arm entry was defined by all four paws crossing the taped grey line. Mice would almost always move to the respective food well once an arm had been entered. If the mouse entered the correct arm, it was allowed to consume the reward. However, if it entered the unrewarded arm, the mouse was removed from the maze once it had seen the empty food well to prevent the contribution of short-term memory errors that can impair its learning (Schmitt et al., 2003). The maze was rotated either clockwise or anticlockwise 120° after each trial so that the mice could not use intra-maze olfactory, tactile or visual cues to help learn the task.

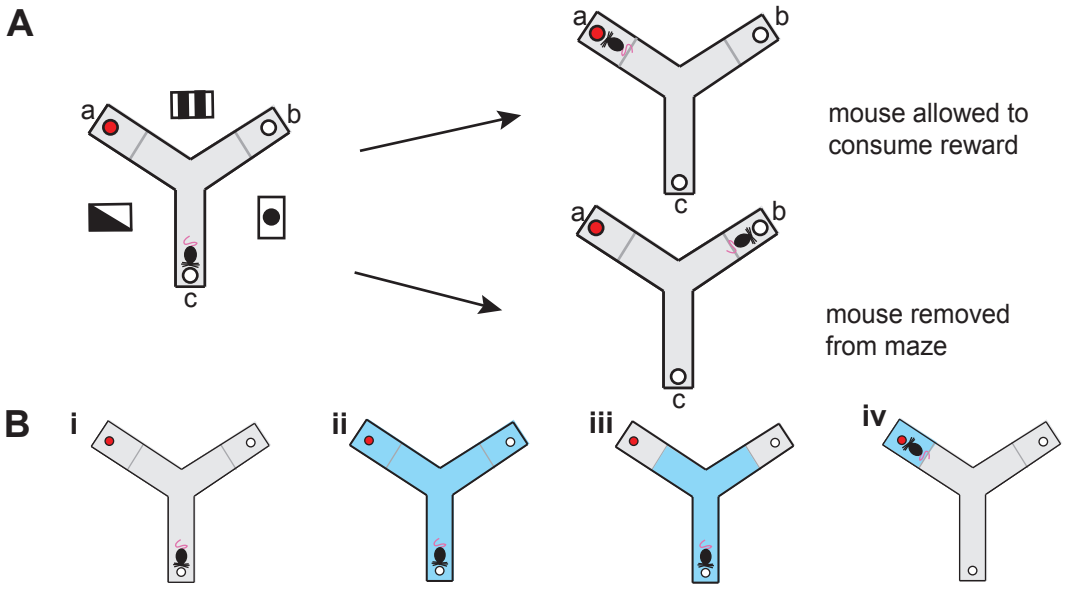


Figure 2.4: Schematic of the appetitive Y-maze task. (A) The appetitive Y-maze task used to test long term spatial memory. Red indicates location of food reward (Arm a). **(B)** Mice were pseudorandomly split into four groups to test four light conditions. Blue indicate illumination for the four light conditions; i: no light, ii: light on throughout the maze, iii: light on only until the reward (grey line) is reached – exploration, and iv: light on only during reward (after grey line has been reached).

2.5.4 Navigation task on an open field

To further evaluate long-term spatial memory, I developed a new appetitive long term memory task on an open field (OF) that more closely mimics exploratory behaviour. The task is similar to a Morris water maze (Vorhees and Williams, 2006) but instead of learning to find the location of a hidden platform under water, mice were trained to reach a reward location in one of the inner quadrants of a circular OF arena using extra-maze spatial cues (Figure 2.5A).

The OF consisted of a green circular board of 110 cm in diameter, bordered by a white 1 cm-high wall, that was elevated 72 cm from the ground. The field was divided into 4 quadrants which were then further divided into an outer and inner section at 55 cm from the centre of the circular field. The inner section of a quadrant was designated the reward location. Approximately one-quarter of each experimental group were assigned to each quadrant to counterbalance for possible quadrant effects. Two plastic food wells (1.5 cm high), secured to the OF with magnets, were positioned at the centre of two opposing inner sections (target and opposite). The testing room was lit with dimmed white light and had distinct painted black and white visual cues made from cardboard and standard furniture. A video camera was mounted at ceiling of the testing room to record the trials and a video tracking software (SMART, Panlab, Spain) was used to record the search paths that each mouse took during each trial.

Mice were food-restricted, fed once daily after habituation or testing and allowed to maintain at least 85% of their free-feeding body weight. Mice were habituated on the open field and food reward (sweentened condensed milk diluted 50:50 in water) in a different room to the testing room. Mice began the task facing outwards in the outer section of either the adjacent left or right quadrant relative to the target quadrant. Each mouse received ten trials per day for 8-12 consecutive days. On the last day of the place learning period, the food reward was given after the mice had entered the inner section of the target

quadrant as a control for mice locating reward by odour (P.C.B.). On each day, they had five starts from the left of the target quadrant and five starts from the right in a pseudorandom order with no more than three consecutive starts from the left or right. Mice were removed from the testing arena if they approached the empty well or if they remained stationary for more than 1 minute or if they did not reach the target or opposite quadrant in 2 minutes. If mice reached the correct target reward area, mice were allowed to consume the food reward and were removed from the testing arena as soon as they moved away from the food well. Between each trial, the OF was rotated 90^o clockwise or anti-clockwise so that mice could not use intra-maze cues to help learn the task.

For testing with ChAT-Ai40D mice, mice were split into two groups (i: 'light on' throughout duration of trial; ii: 'light off', i.e. no light stimulation). For testing with DAT-Ai32 mice and their WT control mice, they were randomly assigned to three groups (i: food reward only; ii: food reward and light stimulation (50 ms-long pulses at 10 Hz) if mice reached the reward location; iii: light stimulation once the mice have reached the inner section of the target quadrant; Figure 2.5B). The target reward location assignments were counterbalanced such that at least one mouse of each experimental group were designated to each quadrant. For mice receiving light stimulation only, if they reached the correct reward location area, mice received light stimulation for 20 seconds or until they moved away from the reward area, whichever occurred first.

After the place learning period, the target reward location was switched to the opposite quadrant and the training was repeated for 12 consecutive days to test reversal learning (Figure 2.5C).

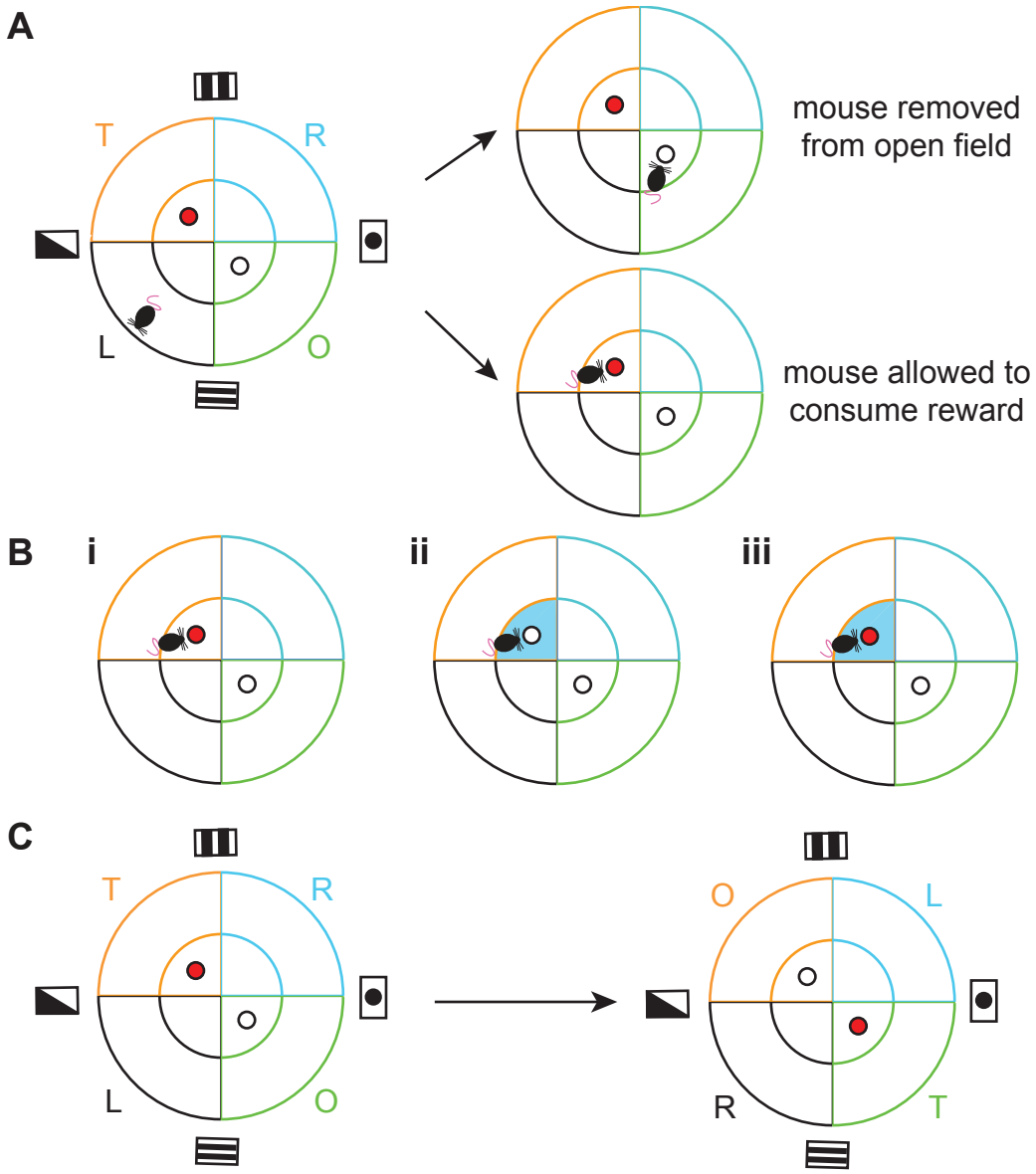


Figure 2.5: Schematic of the open field navigation task. (A) An appetitive open field navigation task. T = target quadrant; O = opposite; L = left adjacent; R = right adjacent. **(B)** ChAT-Ai32 mice were split into three groups: i: food only; ii: light stimulation only; iii: food and light stimulation. Blue shading indicate light stimulation. **(C)** For reversal learning, the reward location was moved to the opposite quadrant while the visual cues in the room remained at the same locations.

2.6 Behavioural data analysis

Statistical analyses were performed using R, SPSS or Prism GraphPad and significance was assessed by repeated measures one-way ANOVA with a within subject factor of light stimulation/delay, one-way ANOVA with a between subjects factor of light stimulation groups, one-sample *t*-test, two-way ANOVA with two between subject factors of genotype and light stimulation (each with two to four levels), one/two-tailed Mann-Whitney test (a non parametric test when the sample sizes were small or when the data did not pass the D'Agostino and Pearson omnibus normality test) or a mixed design ANOVA when there was a mixture of between subjects and within subjects factors. If significance was found, further analysis was performed using the Tukey post hoc test. Significant interactions were explored by simple main effects. All values are given as mean ± S.E.M., and numbers (n) refer to the number of animals.

2.7 Immunohistochemistry

In order to verify the expression of channelrhodopsin or archaerhdopsin and placement of implants, all mice used in behavioural experiments were anaesthetised by intraperitoneal injection of pentobarbital (533 mg/kg) and then transcardially perfused with phosphate-buffered saline (PBS) followed by paraformaldehyde (PFA). Brains were post-fixed for 24-48 hours at 4°C in PFA, then rinsed and subsequently kept in 30% (w/v) sucrose in PBS for at least 24 hours, or until the brain had sunk to the bottom of the holding tube, indicating that sufficient sucrose has infiltrated the brain. 30 μm-thick coronal sections along the entire dorsal-ventral axis of the MS/VTA to hippocampus were cut using a microtome (Spencer Lens Co., Buffalo, U.S.A.) and divided into six series.

For ChAT-Ai32 and ChAT-Ai40D animals, to verify expression of channelrhodopsin fused with the eYFP tag or archaerhdopsin fused with the eGFP tag and visualise the location of cholinergic neurons, one of the series was selected and immunostained for eYFP and ChAT. ChAT catalyses the transfer of an acetyl group to choline to form acetylcholine. ChAT is in high concentration in all cholinergic cells throughout the brain, making it a useful marker for cholinergic neurons (Hedrick et al., 2016; Hoover et al., 1978; Berman et al., 1953). Sections were rinsed for 6×5 minutes in PBS and incubated for 1 hour in a blocking solution comprising of PBS with 0.3% (w) Triton X-100 and 5% (w) donkey serum (Abcam) containing 1% (w/v) bovine serum (Sigma). They were then incubated for 15 hours at 4 °C in blocking solution containing anti-GFP (chicken, 1:1000, Abcam) and anti-ChAT (goat, 1:500, Milipore) antibodies. The sections were then rinsed for 6×5 minutes in PBS, then incubated for 2 hours in blocking solution containing Alexa 488-labelled secondary antibody (goat anti-chicken; 1:1000, Abcam) and Alexa 594-labelled secondary antibody (donkey anti-goat; 1:1000, Abcam) at room temperature. After 6×5 minutes rinse, the sections were mounted in Fluoroshield with DAPI (Sigma).

Similar procedures were used to verify expression of ChR2 fused with the eYFP tag and visualise the location of dopaminergic neurons in DAT-Ai32 mice. However, instead of immunostaining for ChAT, DAT-Ai32 slices were immunostained for tyrosine hydroxylase (TH), the first rate-limiting enzyme involved in the biosynthesis of dopamine from tyrosine, a useful marker for dopaminergic neurons (Daubner et al., 2011; White and Thomas, 2012). The primary antibodies used were anti-GFP (chicken, 1:1000, Abcam) and anti-TH (mouse, 1:1500, Milipore) and the secondary antibodies used were Alexa 488-labeled secondary antibody (goat anti-chicken; 1:1000, Abcam) and Alexa 568-labelled secondary antibody (donkey anti-mouse; 1:1000, Abcam).

Fluorescence images to verify expression of the eYFP/GFP tag and to visualise ChAT or TH labeled neurons were taken with a Leica microsystems SP8 confocal

microscope using the 10× and 20× lens and acquired with Leica Microscope Imaging Software. The number of eYFP+/GFP+ and ChAT+/TH+ cells were quantified manually.

In order to identify the placements of the implants for each mouse, sections containing evidence of the implant were selected from each of the six series and these were mounted in Fluoroshield (Sigma). These sections were viewed in bright-field mode using a Zeiss Axioskop 2 microscope with a 5× lens. The location at which the implant appeared the deepest was determined and used to plot the implant location on the Mouse Brain Atlas (Franklin and Paxinos, 2007).

Chapter 3

Effects of cholinergic or dopaminergic modulation on hippocampal local field potentials

3.1 Introduction

Brain oscillations are rhythmic neural activity that can be readily recorded as local field potentials (LFP). These oscillations are driven by synchronised activity of large ensembles of neurons and this synchronous activity is important for the formation of functional networks that carry out complex cognitive operations (Colgin, 2016). One such cognitive operation is the formation of memory (Düzel et al., 2010). At the cellular level, memories are thought to be encoded in distributed groups of co-active neurons (Hebb, 1949) and brain oscillations are thought to facilitate the coordination of distributed neuronal activity during memory formation.

Numerous studies have demonstrated the importance of the hippocampus for spatial and episodic memory (Scoville and Milner, 1957; Squire and Zola-Morgan, 1991; Kesner, 2007), and it is therefore an ideal structure to investigate how brain oscillations affect memory processes. Furthermore, the hippocampus comprises of densely packed neurons that can generate large oscillatory activity and as such, much research has been carried out in the hippocampus to investigate the role of brain rhythms in memory.

The hippocampus has various types of brain rhythms including *theta* oscillations (∼4–12 Hz), SWR events (∼150–250 Hz ripples superimposed on slow ∼0.01–2 Hz sharp waves) and *gamma* oscillations (∼20–100 Hz). There is general consensus that theta rhythms facilitate the processing of incoming sensory information and encoding of new information (O'Keefe et al., 1993; Colgin, 2013). On the other hand, SWRs promote stabilisation of the cognitive map and consolidation of memories (Girardeau et al., 2009; Ego-Stengel and Wilson, 2010). The functional significance of gamma oscillations is much less clear.

Modulation by subcortical neuromodulators can shift brain rhythms that thereby affect various functions such as learning and memory. ACh is associated with hippocampal theta oscillations, setting favourable conditions for memory encoding. Conversely, reduced levels of ACh facilitate the spread of excitation in the recurrent CA3 network and lead to synchronous SWRs (Marighetto et al., 1993; Hasselmo and Mcgaughy, 2004). The major source of cholinergic input to the hippocampus comes from the MS/diagonal band of Broca. The MS contains 3 main types of neurons: slow-firing cholinergic neurons, fast-firing and burst-firing GABAergic neurons and cluster-firing glutamatergic neurons that may each distinctively contribute to hippocampal rhymicity (Serafin et al., 1996; Sotty et al., 2003; Simon et al., 2006). In particular, in unanaesthetised rats, GABAergic neurons display rhythmic bursting activity during wakefulness or rapid eye movement (REM) sleep with hippocampal theta. During slow-wave sleep, burst-firing neurons take on a tonic firing. In contrast, cholinergic neurons have a slow-firing rate regardless of the state of the sleep/wake cycle (Lee et al., 2005; Simon et al., 2006; Hangya et al., 2009).

Nevertheless, numerous studies have implicated the critical role of MS cholinergic neurons in theta oscillations; inactivation of the MS eliminates hippocampal theta oscillations (Lawson and Bland, 1993) and leads to severe spatial memory deficits (Winson, 1978; Leutgeb and Mizumori, 1999). Similarly, lesions of septal cholinergic neurons by toxins reduces the power of theta, but not its frequency (Lee et al., 1994; Buzsáki, 2002; Li et al., 2007). Consequently, it was proposed that ACh has a permissive role in theta generation, affecting its power, rather than frequency.

Similarly, activating ACh receptors via the application of cholinergic agonists can induce gamma oscillations in the CA3 *in vitro* (Fisahn et al., 1998), and in CA1 in the absence of CA3 input (Pietersen et al., 2014). In contrast, inhibition of cholinergic signalling disrupts phase coupling between gamma and theta (Colgin et al., 2009; Newman et al., 2013). Moreover, a recent study has shown

that in anaesthetised and freely moving mice, activation of septal cholinergic neurons consistently suppresses SWRs (Vandecasteele et al., 2014).

Not much is known about the effects of DA on hippocampal network activity *in vivo*. *In vitro* studies have shown that dopaminergic effects on hippocampal network oscillation is highly dependent on how oscillations are induced. Application of DA increases the power of stimulation-induced gamma oscillations while the power of carbachol- or kainate-induced gamma oscillations is decreased in the presence of DA (Ahveninen et al., 2000; Ma and Leung, 2000; Demiralp et al., 2007; Pinault, 2008; Ehrlichman et al., 2009). This is perhaps unsurprising given the heterogeneity of DA receptors and the heterogeneity of gamma oscillations. DA has also been implicated in hippocampal theta oscillations where depletion of DA decreases theta activity while injection of DA can increase hippocampal theta activity.

3.1.1 Aim

Although there is much indirect evidence supporting the role of septal cholinergic neurons in modulating the power of theta oscillations, direct evidence is scarce. Their effects on gamma oscillations is also unclear. Moreover, the effects of VTA dopaminergic neurons on theta, gamma oscillations and SWRs are even less well described.

To address these issues *in vivo*, I used acute optogenetic activation or silencing of either cholinergic or dopaminergic neurons and examined its impact on hippocampal LFP of urethane anaesthetised mice. Optogenetic tools allow regional- and cell type-specific activation or silencing of neurons on a millisecond timescale and as such, offer a precise method to directly interrogate the involvement of the cholinergic or dopaminergic systems in hippocampal brain rhythms (Witten et al., 2011; Vandecasteele et al., 2014; Steinberg et al.,

2013; Hedrick et al., 2016). Optogenetic activation can be achieved with the light-sensitive cation channel *channelrhodopsin-2* (ChR2); upon activation with blue light, depolarising currents are rapidly induced which activate the neurons (Boyden et al., 2005). In contrast, optogenetic silencing can be carried out with the light-driven outward proton pump *archaerhodopsin-3* (from the *Halorubrum* strain TP009 - ArchT); upon activation with yellow-green light, it pumps protons out of the cells and thus hyperpolarises and silences them (Han et al., 2011).

Given the importance of the cholinergic system in theta rhythms (Vandecasteele et al., 2014), I hypothesised that activation of cholinergic neurons would switch brain states from slow waves (during which SWRs dominate) to theta oscillations, while cholinergic inactivation would enhance SWRs instead. Gamma oscillations have also been demonstrated to depend on cholinergic signalling *in vitro* (Hajos et al., 2004) and thus I hypothesised that gamma oscillations would also be enhanced by cholinergic activation *in vivo*. Not much is known regarding the effects of DA on hippocampal LFP, but several studies *in vitro* have implicated DA in the modulation of various types of gamma oscillations, especially in disease such as schizophrenia, and therefore, I hypothesised that gamma oscillations would be significantly impacted by dopaminergic activation.

To test these hypotheses, I recorded hippocampal LFP, specifically examining oscillations in the theta, slow (sharp waves) and gamma band while activating cholinergic neurons in the MS, dopaminergic neurons in the VTA or inactivating cholinergic neurons in the MS. However, prior to addressing these issues, it is critical to validate the transgenic mouse models used. I had to ensure that expression of optogenetic tools are cell-type specific and that these tools have physiological effects.

Three types of transgenic mice were used in this thesis: (i) *ChAT-Ai32* mice which express ChR2 tagged with a fluorescent protein (ChR2-eYFP) in cholin-

ergic neurons; (ii) *ChAT-Ai40D* mice which express GFP (eGFP)-tagged ArchT fusion protein in cholinergic cells and (iii) *DAT-Ai32* mice which express ChR2-eYFP in dopaminergic neurons. To confirm that expression of ChR2 or ArchT was specific to their designated cell types, I performed immunohistochemistry to determine expression selectivity prior to hippocampal LFP recordings. To ensure that the opsins have a physiological effect, I also performed *in vivo* multi-unit activity (MUA) recordings in the MS or the VTA of urethane-anaesthetised mice. To further verify that the effects of cholinergic activation on hippocampal rhythms were not only limited to anaesthetise animals, two ChAT-Ai32 mice were also implanted with an optic fibre cannula and chronic electrodes for freely moving recordings.

3.2 Cholinergic activation

To examine the effects of the septo-hippocampal cholinergic input, I used transgenic mice expressing light-activated cation channel ChR2, tagged with a fluorescent protein (ChR2-eYFP) under the control of the ChAT promoter. These mice were referred to as ChAT-Ai32 mice.

3.2.1 ChR2-eYFP expression in ChAT-Ai32 mice is selective in cholinergic neurons

To verify the level of transgene expression and selectivity, ChAT-Ai32 mice were perfused and coronal sections of the MS and hippocampus were made for immunohistochemistry. Sections were stained for ChAT, a specific marker for cholinergic neurons (Houser et al., 1983; Oda, 1999) and YFP (to visualise localisation of ChR2-eYFP). DAPI was also used to visualise cell nuclei (Tarnowski et al., 1991). Immunostaining revealed that ChAT-Ai32 mice showed selective eYFP-positive staining in MS ChAT neurons (Figure 3.1A). Of 111

ChAT-immunopositive neurons in the MS, 98 (83.13%) were also positive for ChR2-eYFP (YFP^+ChAT^+). There was no ChR2-eYFP-immunopositive and non ChAT-immunopositive cells (YFP^+ChAT^-), suggesting selective expression of ChR2-eYFP in ChAT cells. Hippocampal sections also revealed $ChR2^+$ fibres in the hippocampus are also $ChAT^+$, suggesting ChR2-expressing cholinergic inputs in the hippocampus (Figure 3.1B, C).

3.2.2 Cholinergic activation increased multi-unit activity in the MS

To confirm that it was possible to activate putative cholinergic neurons by illumination of the MS, I first performed MUA recordings in the MS of urethane-anaesthetised ChAT-Ai32 mice. I stripped an optical fibre to the 200 μm core and tightly bundled it to a 1 MΩ tungsten monopolar recording electrode. The tip of the recording electrode protruded approximately 0.5 mm below the tip of the optical fibre to ensure that the recorded cells received illumination but were not damaged by the optical fibre.

The application of 50 ms-long pulses correlated with an increase in MUA (baseline spike frequency: 7.56 $\pm$ 0.58 spikes/s vs. 'light on' spike frequency: 22.69 $\pm$ 0.85 spikes/s, two-tailed paired *t*-test: $p<0.001$; n=6 recordings from 2 mice; Figure 3.2), suggesting activation of neurons, most likely cholinergic neurons, in response to light stimulation. The ideal experiment to prove that the light does indeed induce the activation of cholinergic neurons is to do juxtacellular recordings followed by electroporation of the cell (Pinault, 2011). Nevertheless, the combination of immunohistochemistry and MUA recordings strongly suggest that the increased MUA activity during light stimulation is due to activation of cholinergic neurons.

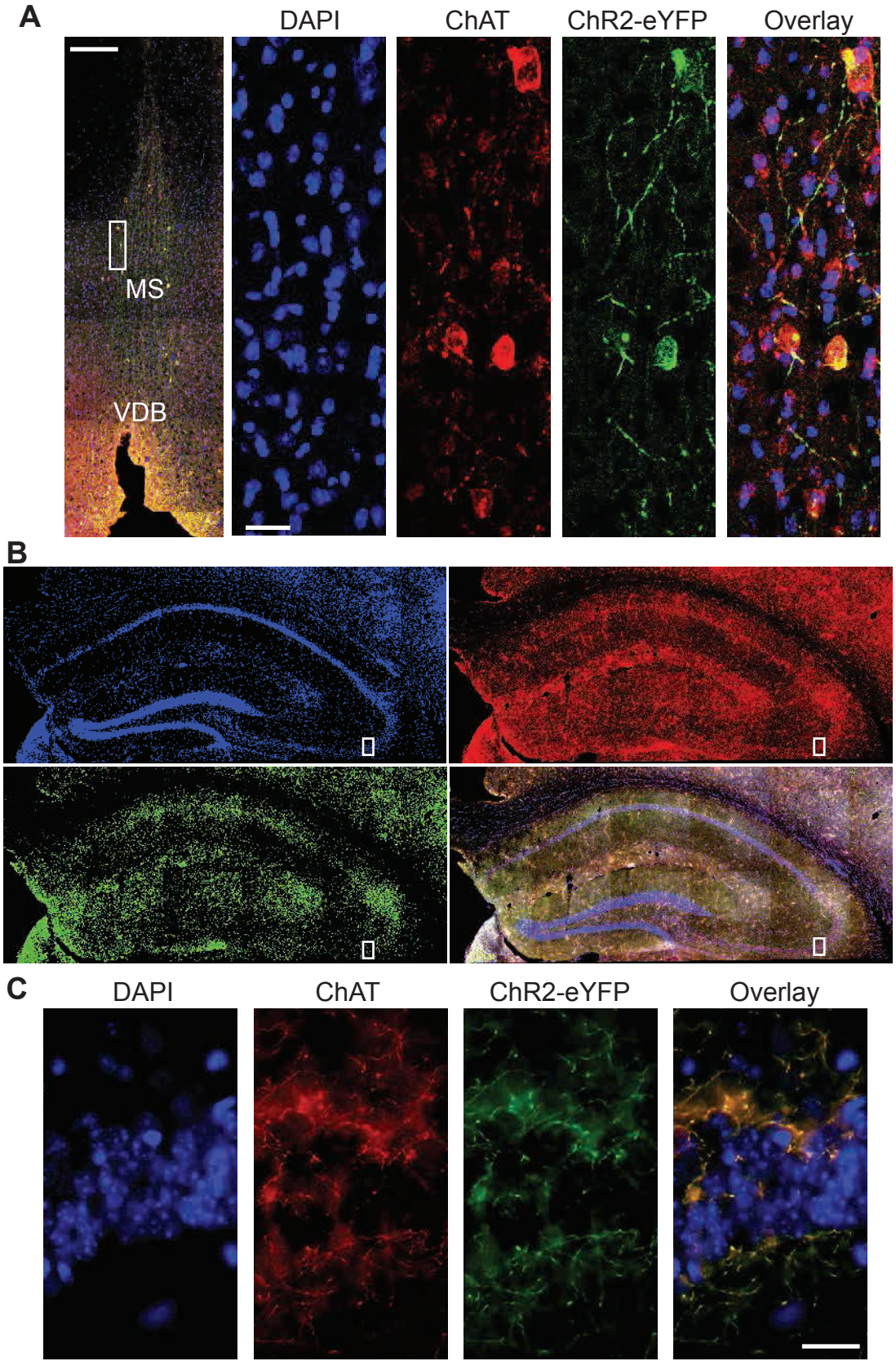

A
DAPI
ChAT
ChR2-eYFP
Overlay
MS
VDB
B
C
DAPI
ChAT
ChR2-eYFP
Overlay

Figure 3.1: Selective expression of ChR2-eYFP in cholinergic cells of ChAT-Ai32 mice. (A) Left-most panel: Overlay of DAPI, ChAT and eYFP-positive immunostaining in a coronal section of the medial septum (MS) in a ChAT-Ai32 mouse. Scale bar: 500 μm. VDB, ventral diagonal band. Right panels: higher magnification of the MS (rectangle in left panel), triple immunostaining of DAPI (blue, second left panel), ChAT (red, middle) and eYFP (green, second right panel), showing their colocalisation (overlay, right). Scale bar: 50 μm. **(B)** DAPI (blue), ChAT (red) and eYFP (green)-positive immunostaining in a coronal section of the hippocampus in a ChAT-Ai32 mouse. Scale bar: 500 μm **(C)** High magnfication of the CA3 (white box in B), triple immunostaining of DAPI (blue, left-most panel), ChAT (red, second-left panel) and eYFP (green, second-right panel), showing their colocalisation (overlay, right). Scale bar: 25 μm

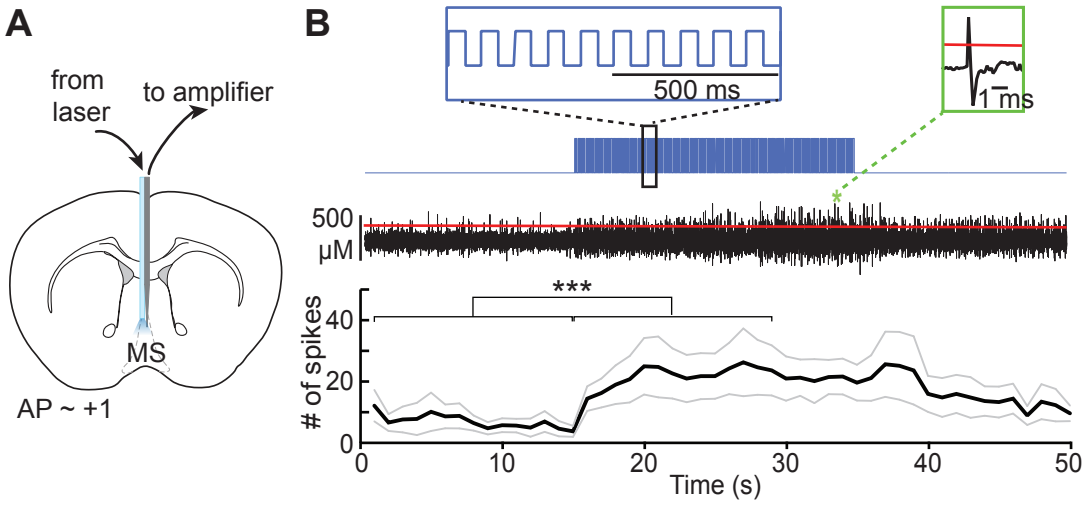


Figure 3.2: Multi-unit activity in the MS increases with light stimulation in ChAT-Ai32 mice. (A) Schematic of the experimental set up. **(B)** A sample trace of the recording. Top: the stimulation protocol (blue) which begins at 15 s. Inset shows an example of the 50 ms-long, 10 Hz square stimulation pulses. Middle: an example recording trace, green box inset shows an example unit recorded. The mean frequency of spiking is illustrated in the graph (n=6; bottom). ***p<0.001. Grey lines represent S.E.M.

3.2.3 CA3 LFP under urethane anaesthesia

Urethane was used to anaesthesised the mice as urethane most mimics the natural sleep cycle during the induced sedative state (Kramis et al., 1975; Clement et al., 2008; Pagliardini et al., 2013). In natural sleep, brain activity follows a stereotyped cycle of transitions that typically progress from wakefulness to light non-REM sleep and eventually to deep non-REM, before returning from non-REM sleep stages into REM sleep. This cycle repeats for the entire sleep epoch. During deep non-REM sleep, also called slow wave sleep, brain activity is associated with slow, large amplitude waves (Steriade et al., 1993; Dang-Vu et al., 2008). In contrast, brain activity during REM sleep is characterised by low amplitude, faster oscillating rhythms that are similar to rhythms during wakefulness (Saper et al., 2010).

The majority of anaesthesia induces a unitary level of brain activity that is of large amplitude and low frequency, similar to slow wave sleep (Tung and

Mendelson, 2004). However, under urethane anaesthesia, rodents maintain long durations of alternating cycles of brain activity without additional pharmacological manipulations (Clement et al., 2008; Pagliardini et al., 2013). In urethane-anaesthetised ChAT-Ai32 mice, I recorded hippocampal LFP which cycled between deep sleep-like SWR state and REM-like theta-gamma state (Figure 3.3). Theta-gamma states were also sometimes induced by a continuous tail pinch.

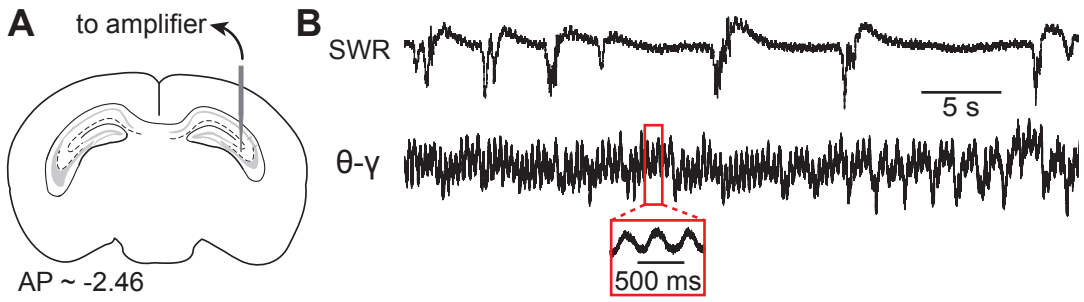


Figure 3.3: Hippocampal LFP oscillated between SWR and theta-gamma states under urethane anaesthesia. (A) Schematic showing the location of the recording electrode in CA3. AP = anterior-posterior **(B)** Examples of hippocampal recordings in urethane-anaesthetised mice. LFP alternated between sharp-wave ripple (SWR; top) and theta-gamma (θ-γ; bottom) states. Inset depicts a 1 s trace.

3.2.4 CA3 responses to MS cholinergic activation during SWR state

Given the alternating states induced by urethane anaesthesia, LFP recordings were performed in both states. Sleep-like SWR state was defined as the predominant occurrences of slow, sharp wave events of less than 2 Hz (Buzsáki, 1986; Ramadan et al., 2009).

During SWR state, I lowered a 1 MΩ tungsten electrode into the CA3 of the hippocampus to record hippocampal LFP. As the frequency, power and amplitude of the oscillatory events can greatly fluctuate over the course of a 45 s-long trace (as can be observed in Figure 3.3), I first recorded 45 s-long traces during

SWR state with no light stimulation and the final 30 s was compared to the first 15 s-baseline as a control. I specifically examined hippocampal slow (and in essence, sharp wave events), theta and slow gamma oscillations by calculating the power of the frequency of interest during light stimulation, calculated as the area under the power spectral density (PSD) curve, normalised to the power of the same frequency of interest during baseline recording. The control recordings were then compared to two other groups of recordings. To determine the effects of cholinergic activation on hippocampal slow oscillations (SO), theta and slow gamma oscillations, a second group of recordings with a 15 s-long baseline followed by 30 s-long of 10 Hz, 25 mW, 473 nm light stimulation were assessed. To verify that these changes (if any) were mediated by cholinergic mechanisms, a third group of recordings were made in some mice that were also intraperitoneally injected with a muscarinic antagonist, scopolamine (2 mg/kg), which did not have a significant effect on baseline oscillatory activity when compared to baseline recordings with no light stimulation (paired *t*-test: SO: $t(4)=1.532$, p=0.2; theta: $t(4)=1.607$, p=0.1833; gamma: $t(4)=0.093$, p=0.93; n=5 pairs of recordings from 5 mice). In these mice, at least 15 minutes after the drug injection, a 15 s-long baseline was recorded, followed by 30 s of 10 Hz, 25 mW, 473 nm light stimulation.

For the control recordings, I saw small fluctuations in the power of SO (0.5–2 Hz; 100±3%; n=7), theta (2–6 Hz; 90±9%; n=7) and slow gamma (20–40 Hz 84±13%; n=7) oscillations. To more closely examine the theta and SO relationship, I also calculated the theta/SO ratio (96±9%; n=7). Activation of cholinergic neurons in the MS during SWR state changed hippocampal LFP from slow, irregular activity to rhythmic theta oscillations. The theta band (2–6 Hz) increased by 325 ± 85% (n=10), which was blocked by scopolamine (95 ± 10%; n=8; Figure 3.4). An analysis of variance (ANOVA) ran to compare group means (i: no light, ii: 30 s light stimulation and iii: 30 s light stimulation +scopolamine) revealed a significant difference in the means of the groups ($F(2,22)=5.43$, p=0.012). Assuming local homogeneity, Gabriel post-hoc test

revealed significant differences between the no light and 30 s light stimulation ($p=0.032$) and the 30 s light stimulation and +scopolamine groups ($p=0.03$).

In contrast, SO (0.5–2 Hz) were suppressed to 58±10% (n=10) and this effect was also blocked by scopolamine (101±6%; n=8; Figure 3.4). One-way ANOVA revealed a significant effect of the light stimulation on the power of SO and presence of scopolamine ($F(2,22)=10.31$, $p=0.0007$). Gabriel post-hoc test revealed significant differences between the no light and 30 s light stimulation ($p=0.004$) and the 30 s light stimulation and +scopolamine groups ($p=0.002$), suggesting that cholinergic effects on SO is primarily mediated by muscarinic receptors.

As a result, the theta/SO ratio increased significantly with stimulation (581±112%; n=10), which was blocked by scopolamine (98±14%; n=8; Figure 3.4). The overall ANOVA showed that the different stimulation groups exhibited significant differences ($F(2,22)=13.99$, $p=0.0001$). Individual group comparisons showed that the no light and 30 s light stimulation ($p=0.001$) and the 30 s light stimulation and +scopolamine groups ($p=0.000$) differed significantly. These results are similar to those reported in Vandecasteele et al. (2014).

Nested within the theta rhythm are faster oscillations called gamma oscillations. I more closely examined those in the 20–40 Hz frequency range (slow gamma) as they are most prominent in the CA3. I found that septal cholinergic activation increased the power of slow gamma oscillations by 225±48% (n=10). This increase was absent in the presence of scopolamine (89±5%; n=8; Figure 3.5). The overall one-way ANOVA indicated that the groups showed significant differences ($F(2,22)=5.95$; $p=0.0086$). Individual group comparisons revealed significant differences between the no light and 30 s light stimulation ($p=0.023$) and the 30 s light stimulation and +scopolamine groups ($p=0.023$).

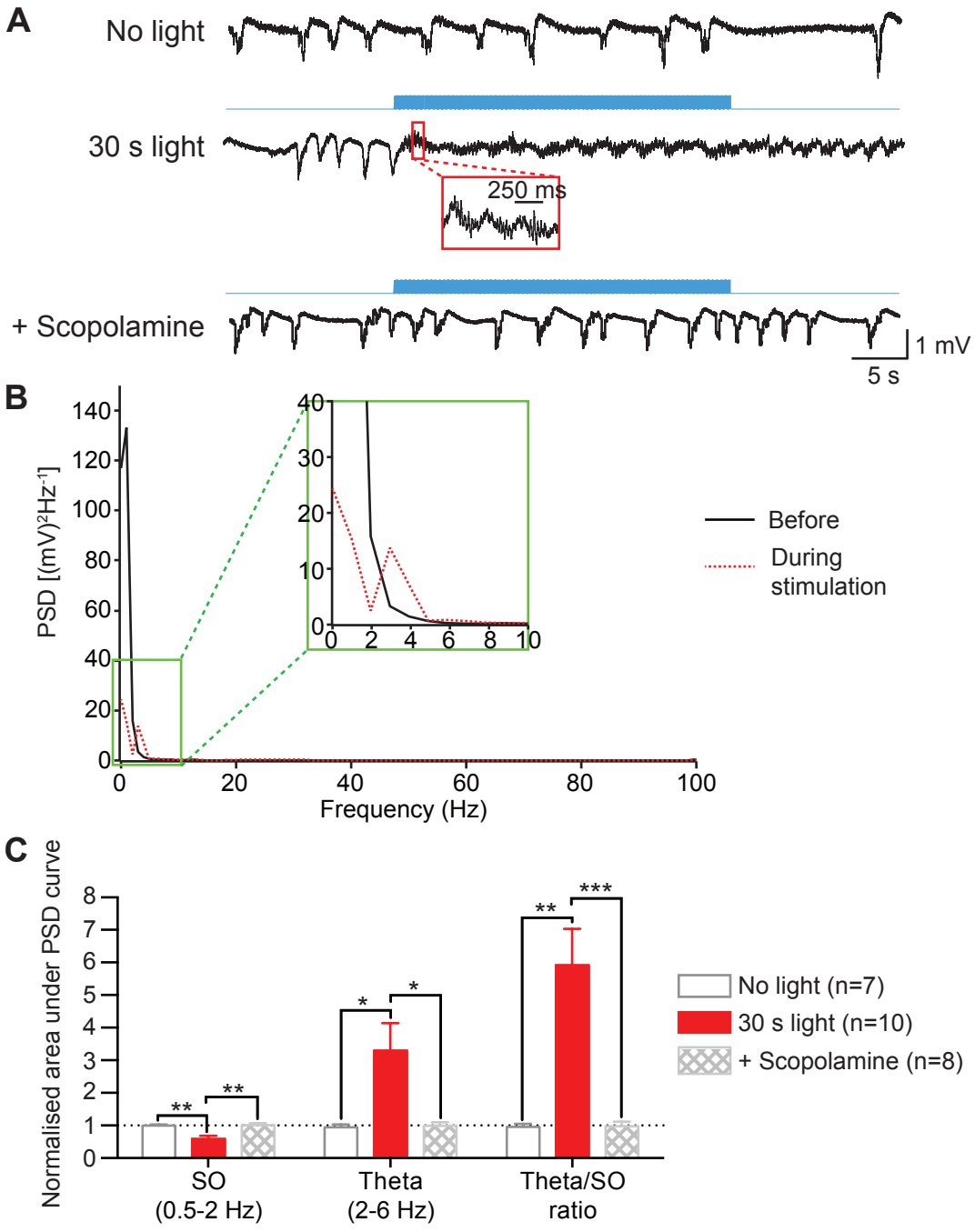

A
No light
30 s light
250 ms
+ Scopolamine
1 mV
5 s
B
PSD [(mV)²Hz⁻¹]
Frequency (Hz)
Before
During stimulation
C
Normalised area under PSD curve
SO (0.5-2 Hz)
Theta (2-6 Hz)
Theta/SO ratio
No light (n=7)
30 s light (n=10)
+ Scopolamine (n=8)

Figure 3.4: MS cholinergic stimulation enhanced power of theta oscillations and suppressed SO during sleep-like SWR state. **(A)** Example traces of hippocampal LFP recordings during SWR state. Top: a baseline trace with no light stimulation. Middle: an example trace with 30 s-long stimulation starting at 15 s after which slow, large sharp wave activity was replaced by faster oscillations that were smaller in amplitude. Inset depicts a 1 s-long trace. Blue bar indicates time with light stimulation. Bottom: an example trace after the injection of a muscarinic antagonist scopolamine (2 mg/kg). **(B)** The PSD curve of an example trace before (black) and during stimulation (red). Inset depicts the frequency range of 0–6 Hz where during stimulation (red), the power of slow oscillations decreased with the emergence of a peak in the theta frequency range (2–6 Hz) during light stimulation. **(C)** Area under the PSD curve for the frequencies of interest for the trace during 30 seconds of the test condition (no light, 30 seconds of light stimulation or 30 seconds of light stimulation in the presence of 2 mg/kg of scopolamine) was calculated and normalised to the same area during baseline recording. Dotted line shows one, indicating no change between the baseline and test recording. *$p<0.05$; **$p<0.01$; ***$p<0.001$.

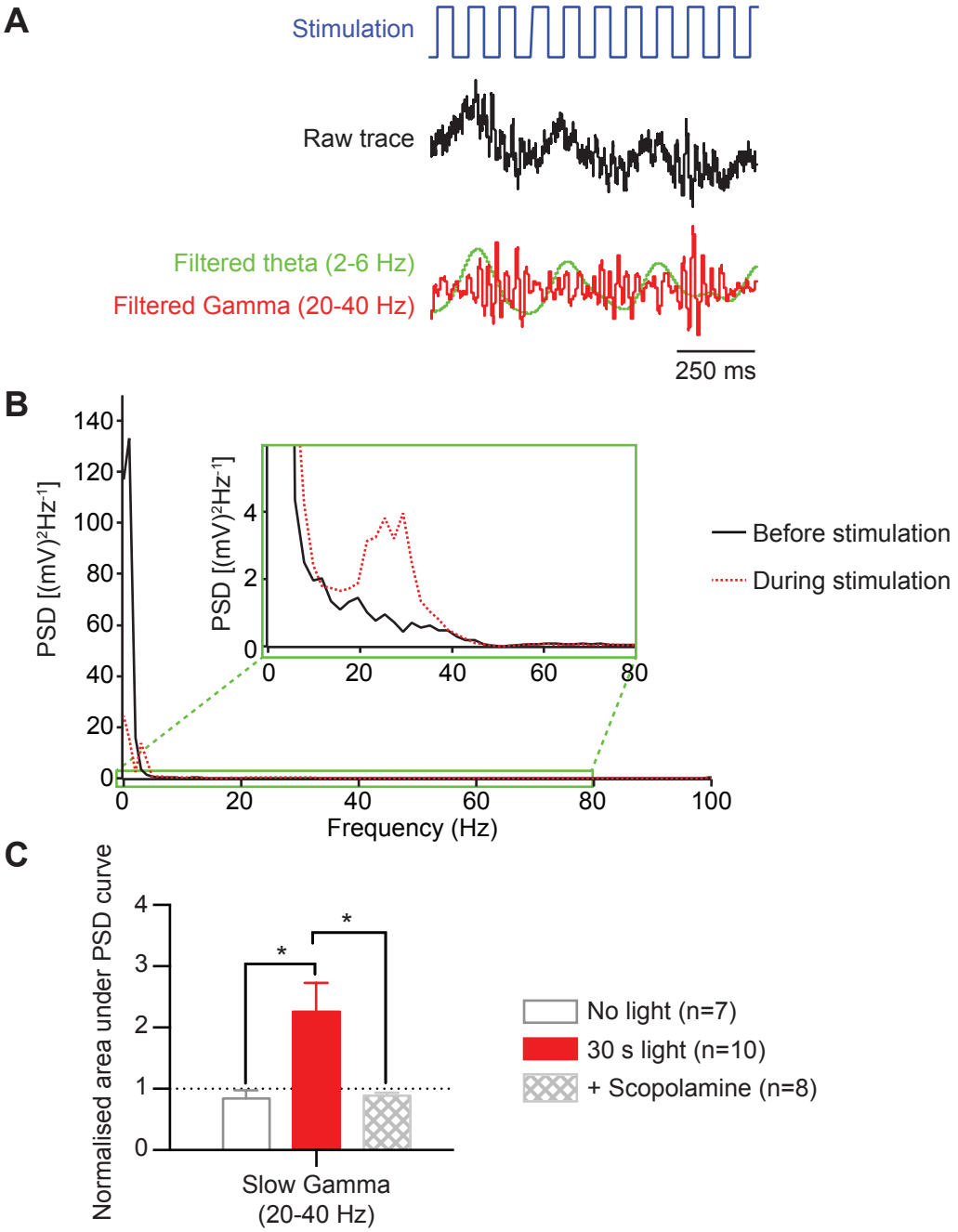

A
Stimulation
Raw trace
Filtered theta (2-6 Hz)
Filtered Gamma (20-40 Hz)
250 ms
B
PSD [(mV)²Hz⁻¹]
Frequency (Hz)
Before stimulation
During stimulation
C
Normalised area under PSD curve
Slow Gamma
(20-40 Hz)
No light (n=7)
30 s light (n=10)
+ Scopolamine (n=8)

Figure 3.5: Power of slow gamma oscillations increased during cholinergic activation. (A) A 1 s example trace (black) during 10 Hz light stimulation (blue). The raw trace was filtered at 2–6 Hz for theta (green) and at 20–40 Hz for slow gamma (red) oscillations. **(B)** The PSD curve of an example trace before (black) and during stimulation (red). Inset depicts the frequency range of 0–80 Hz with the emergence of a peak in the slow gamma frequency range (20–40 Hz) during stimulation. **(C)** Area under the PSD curve for the slow gamma frequency of the trace during 30 seconds of the test condition (no light, 30 seconds of light stimulation or 30 seconds of light stimulation in the presence of 2 mg/kg of scopolamine) was calculated and normalised to the same area during baseline recording. Dotted line shows one, indicating no change between baseline and test recording. *$p<0.05$.

3.2.4.1 Light intensity

To determine the optimal light intensity that would yield the greatest change in the power of the frequencies of interest, I varied the intensity of light delivered at the tip of the optic fibre from 5 to 30 mW. To ensure that there was no fatigue of responses, the sequence of light intensity tested was randomised for different trials. I found that from 15 mW, the power of theta and gamma increased gradually and peaked at 25 mW laser power (Figure 3.6). The suppression of SO reflected a similar pattern.

3.2.4.2 Stimulation frequency

To ensure that the increase in the power of theta and gamma oscillation in the hippocampus was not due to the light stimulation, I varied the frequency of light pulses used to activate the cholinergic neurons from 2 to 20 Hz at 25 mW light intensity. To ensure that there was no fatigue of responses, the sequence of stimulation frequency tested was randomised for different trials. To assess the time and frequency information in the recorded LFP simultaneously, a continuous wavelet transform (CWT) was calculated. I found that the frequency of oscillations in the hippocampus did not follow the frequency of stimulation (Figure 3.7A) and the power of the oscillations did not differ greatly with varying frequencies (Figure 3.7B). This is consistent with the findings reported by Vandecasteele et al. (2014).

3.2.5 CA3 responses to MS cholinergic activation during arousal-like theta-gamma periods

REM sleep is characterised by a strong 2–6 Hz oscillatory activity, known as theta oscillations (Leung, 1984). Nested within theta activity are high frequency gamma oscillations (20-100 Hz; Montgomery et al., 2008). This theta-gamma

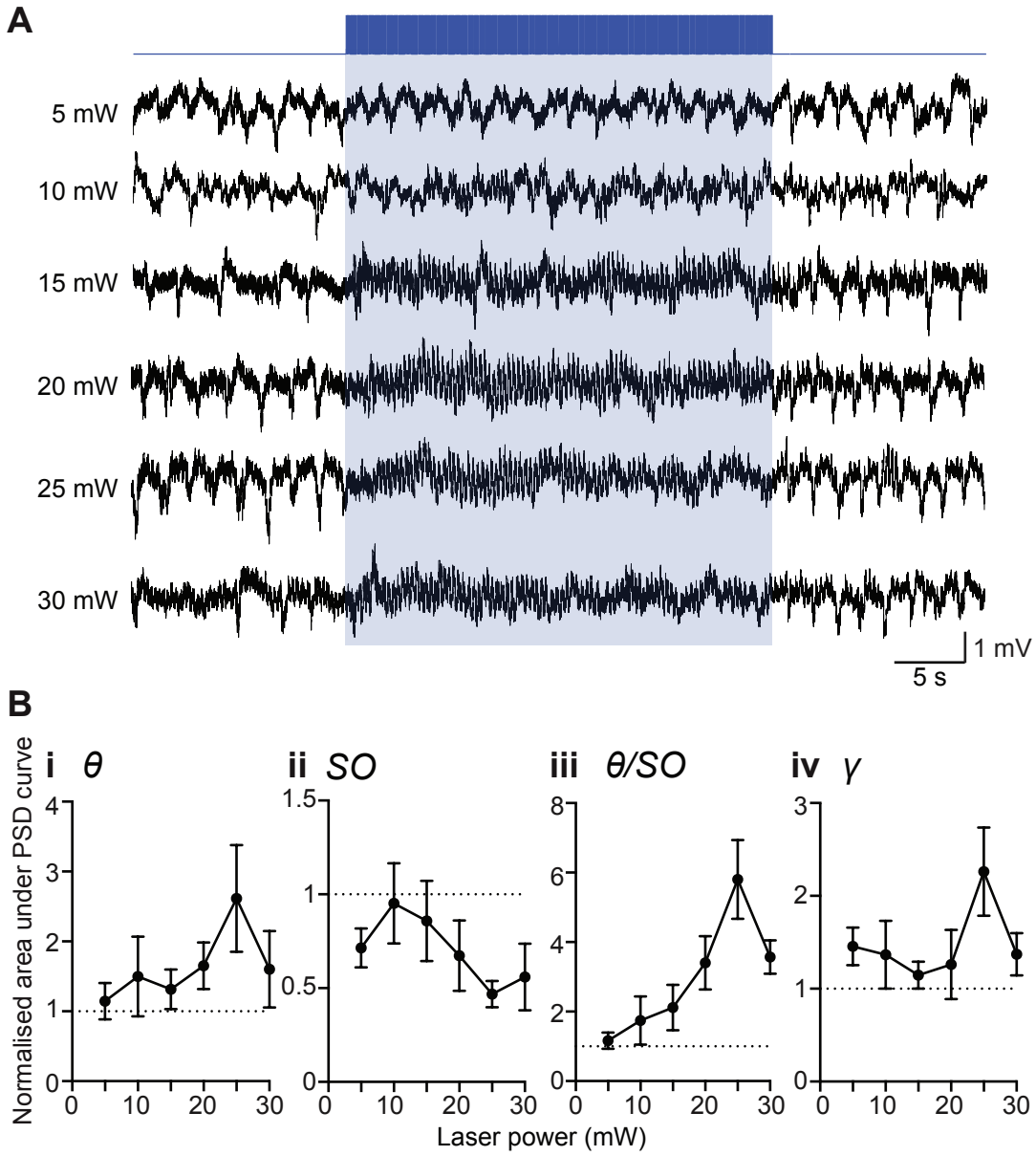


Figure 3.6: Effects of light stimulation peaked at 25 mW laser power. (A) Example hippocampal LFP traces when light stimulation was varied between 5-30 mW. Blue bar indicates light stimulation. **(B)** The mean power ratio was calculated for theta **(i)**, SO **(ii)**, Theta/SO ratio **(iii)** and slow gamma oscillations **(iv)** for light intensity of 5 (n=6), 15 (n=7), 20 (n=7), 25 (n=10) and 30 (n=6) mW. Error bars represent S.E.M.

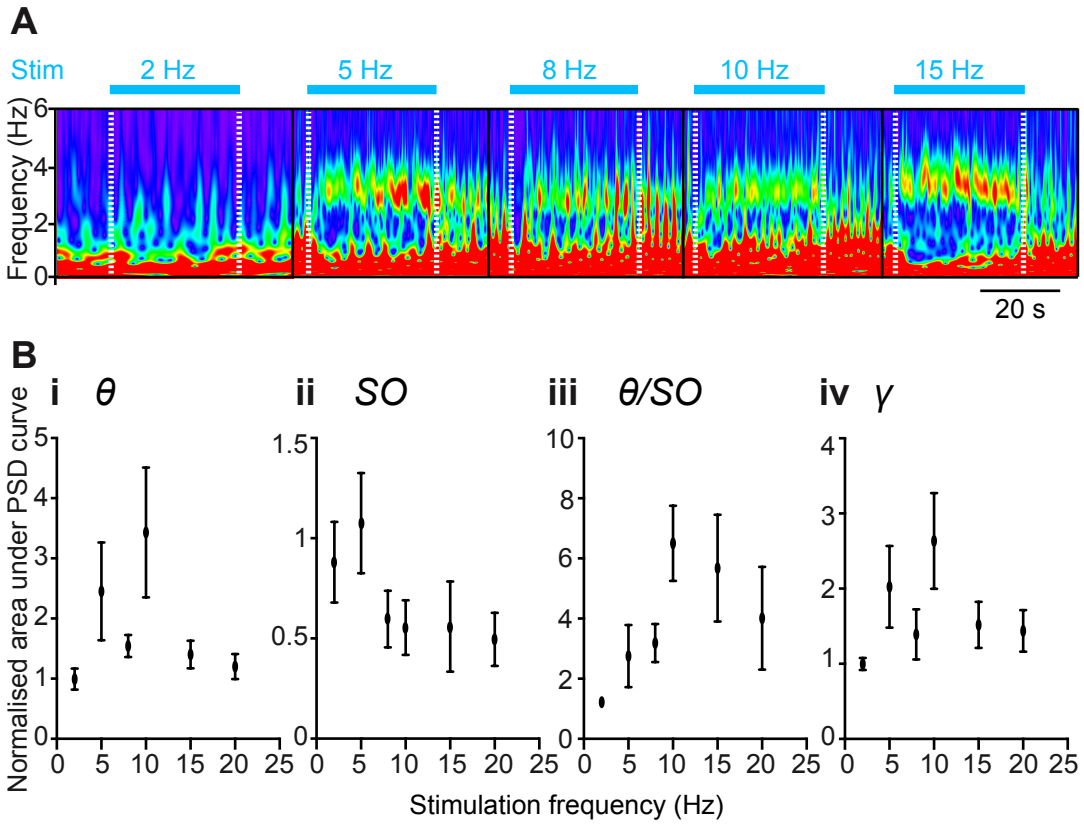


Figure 3.7: Frequency of stimulation did not affect theta frequencies in the CA3. (A) Spectrograms of 5 hippocampal LFP recordings during light pulse stimulation of the MS at 2, 5, 8, 10 and 15 Hz. Black vertical lines indicate separate recordings (one for each stimulation frequency) and white lines indicate the start and end of light stimulation periods. Warmer colours indicate higher magnitude. Blue blocks indicate light stimulation. **(B)** The average power ratio was calculated for theta **(i)**, SO **(ii)**, Theta/SO ratio **(iii)** and slow gamma oscillations **(iv)** for 2 Hz (n=5), 5 Hz (n=6), 8 Hz (n=5), 10 Hz (n=8), 15 Hz (n=6) and 20 Hz (n=5) stimulation frequency. Error bars represent S.E.M.

activity is also observed during awake, exploratory behaviour (O'Keefe et al., 1993; Losonczy et al., 2010).

To examine any differences in the effect of cholinergic activation on hippocampal LFP during sleep-like SWR and arousal-like theta-gamma periods, I also recorded hippocampal LFP in the CA3 during arousal-like theta-gamma state. The switch between brain states often depends on the level of anaesthesia. Alternatively, reliable hippocampal theta can by evoked by continuous tail pinch. However even then, there was often a tendency for brain activity to switch back to SWR (Figure 3.3). As a control, I recorded 45 s-long traces during theta-gamma state with no light stimulation. The final 30 s was compared to the first 15 s. The power of SO (0.5–2 Hz; 143±14%; n=12) increased while slow gamma oscillations (80±9%; n=12) and the theta/SO ratio decreased (82±10%; n=12). The power of theta oscillations remained constant (103±8%; n=12).

Similar to recordings made during SWR state, a second group of recordings with a 15 s-long baseline followed by 30 s-long of 10 Hz, 25 mW, 473 nm light stimulation were also assessed and the differences between these two groups addressed the question of the effects of cholinergic activation on the frequencies of interest during theta-gamma periods. To verify that these changes were mediated by cholinergic mechanisms, a third group of recordings were made in 8 mice that were also intraperitoneally injected with a muscarinic antagonist, scopolamine (2 mg/kg). In these mice, at least 15 minutes following the drug injection, a 15 s-long baseline was recorded, followed by 30 s of 10 Hz, 25 mW, 473 nm light stimulation.

Theta did not change during theta-gamma states during optogenetic stimulation but prevented the tendency of the brain activity to go back to SWR state. SO were also significantly suppressed to 73±15% (n=13). This was absent in the presence of scopolamine (138±21%; n=8; Figure 3.8). The overall ANOVA

showed that the different stimulation groups exhibited significant differenes ($F(2,30)=6.42$, $p=0.0048$). Individual group comparisons revealed that the no light and 30 s light stimulation ($p=0.008$) and the 30 s light stimulation and +scopolamine groups ($p=0.03$) differed significantly.

In contrast to SWR state, there was no significant increase in the power of theta oscillations. Theta oscillations stayed relatively constant at 113±17% (n=13). Scopolamine had little effect on theta power (99±13%; n=8; Figure 3.8). There was no significant difference between the group means ($F(2,30)=0.2618$, $p=0.77$). Stimulation of cholinergic neurons strongly enhanced power of slow gamma oscillations (203±57%; n=13). This increase was blocked by the presence of scopolamine (83±11%; n=8). However, one-way ANOVA did not reveal significant differences between the group means ($F(2,30)=3.308$, $p=0.0503$; Figure 3.8).

Consequently, the Theta/SO ratio significantly increased to 221±39% (n=13), which was blocked by scopolamine (84±17%; n=8; Figure 3.8). One-way ANOVA to compare group means (i: no light, ii: 30 s light stimulation and iii: 30 s light stimulation +scopolamine) revealed a significant difference in the means of the groups ($F(2,30)=8.610$, $p=0.0011$). Assuming local homogeneity, Gabriel post-hoc test revealed significant differences between the no light and 30 s light stimulation ($p=0.002$) and the 30 s light stimulation and +scopolamine groups ($p=0.008$).

3.2.6 Recordings in freely moving animals

Vandecasteele et al. (2014) reported that the effects of septal cholinergic activation differed between anaesthetised and behaving animals. Thus, to ensure that the results observed in anaesthetised recordings are comparable to what happens during a behavioural task, I implanted two ChAT-Ai32 mice each with

a mono fibre-optic cannula (0.22 NA) in the MS, a 1 MΩ platinum-iridium recording electrode in the right CA3 and a reference electrode in the contralateral cortex. The fibre-optic cannula was connected to the laser via a patchcord for light stimulation. The mice were allowed to move freely and a food reward was placed in the middle of the test arena for the mice to mimic consummatory behaviour observed during an appetitive behavioural task.

During consummatory behaviour, hippocampal LFP displayed large irregular activity, similar to those observed during slow wave sleep. To investigate the effect of septal cholinergic activation, brief (50 ms) pulses of 25 mW 473 nm light was delivered via the fibre-optic cannula. MS stimulation suppressed SO to 53±32%, while the theta/SO ratio increased to 358±229% (n=2; Figure 3.9). No recordings were made while the mice were in motion due to noise. A different recording setup would be required to acquire such data.

3.3 Cholinergic inactivation

I used ChAT-Ai40D mice to investigate the effects of cholinergic inactivation on hippocampal LFP. ChAT-Ai40D mice are offspring of the ChAT-Cre line crossed with the Ai40D line. They express eGFP-tagged archaerhodopsin (ArchT-eGFP) in cholinergic cells.

3.3.1 ChAT-Ai40D mice selectively express ArchT-eGFP in cholinergic cells

To verify the level of transgene expression and selectivity, ChAT-Ai40D mice were perfused and coronal sections of the MS and hippocampus were prepared for immunohistochemistry. Similar to sections from ChAT-Ai32 mice, ChAT-Ai40D sections were stained for ChAT, GFP (to visualise localisation of ArchT-eGFP)

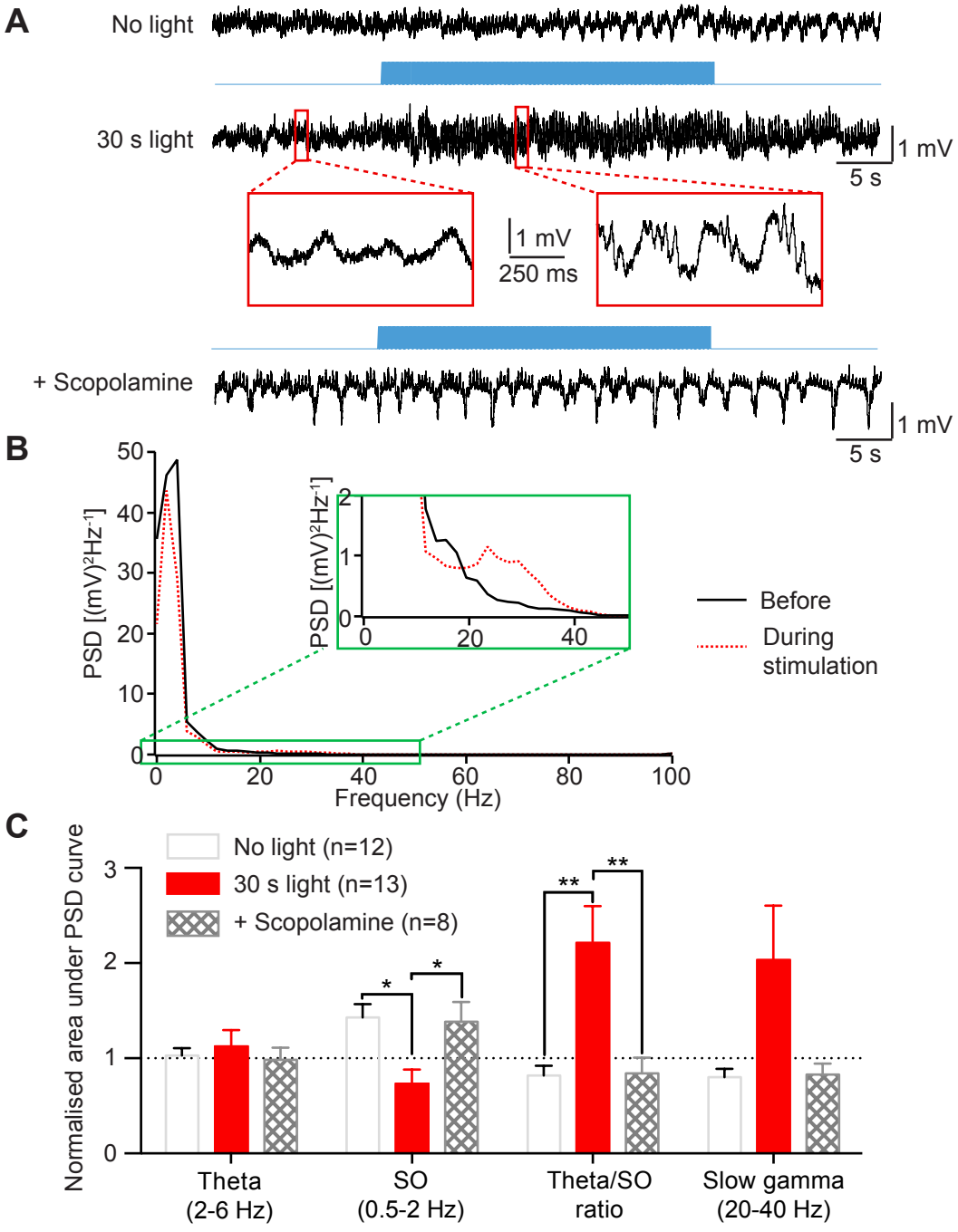

A
No light
30 s light
1 mV
5 s
1 mV
250 ms
+ Scopolamine
1 mV
5 s
B
PSD [(mV)²Hz⁻¹]
Frequency (Hz)
Before
During stimulation
C
Normalised area under PSD curve
No light (n=12)
30 s light (n=13)
+ Scopolamine (n=8)
Theta (2-6 Hz)
SO (0.5-2 Hz)
Theta/SO ratio
Slow gamma (20-40 Hz)

Figure 3.8: During theta-gamma state, stimulation of cholinergic neurons suppressed the tendency of brain activity to go back to SWR state and increased theta/SO ratio. (A) Example traces of hippocampal LFP recordings during theta-gamma state. Top: a baseline trace with no light stimulation. Middle: an example trace with 30 s-long stimulation starting at 15 s. Inset depicts a 1 s-long trace before and during light stimulation. Bottom: an example trace after the injection of a muscarinic antagonist scopolamine (2 mg/kg). Blue bars indicate time with light stimulation. **(B)** The PSD curve of an example trace before (black) and during stimulation (red). Inset depicts the frequency range of 0–50 Hz where during stimulation (red), the power of gamma oscillations increased (20–40 Hz). **(C)** Area under the PSD curve for the frequencies of interest for the trace during 30 seconds of the test condition (no light, 30 seconds of light stimulation or 30 seconds of light stimulation in the presence of 2 mg/kg of scopolamine) was calculated and normalised to the same area during baseline recording. Dotted line shows 1, indicating no change before and after test condition. Error bars represent S.E.M. *$p<0.05$; **$p<0.01$.

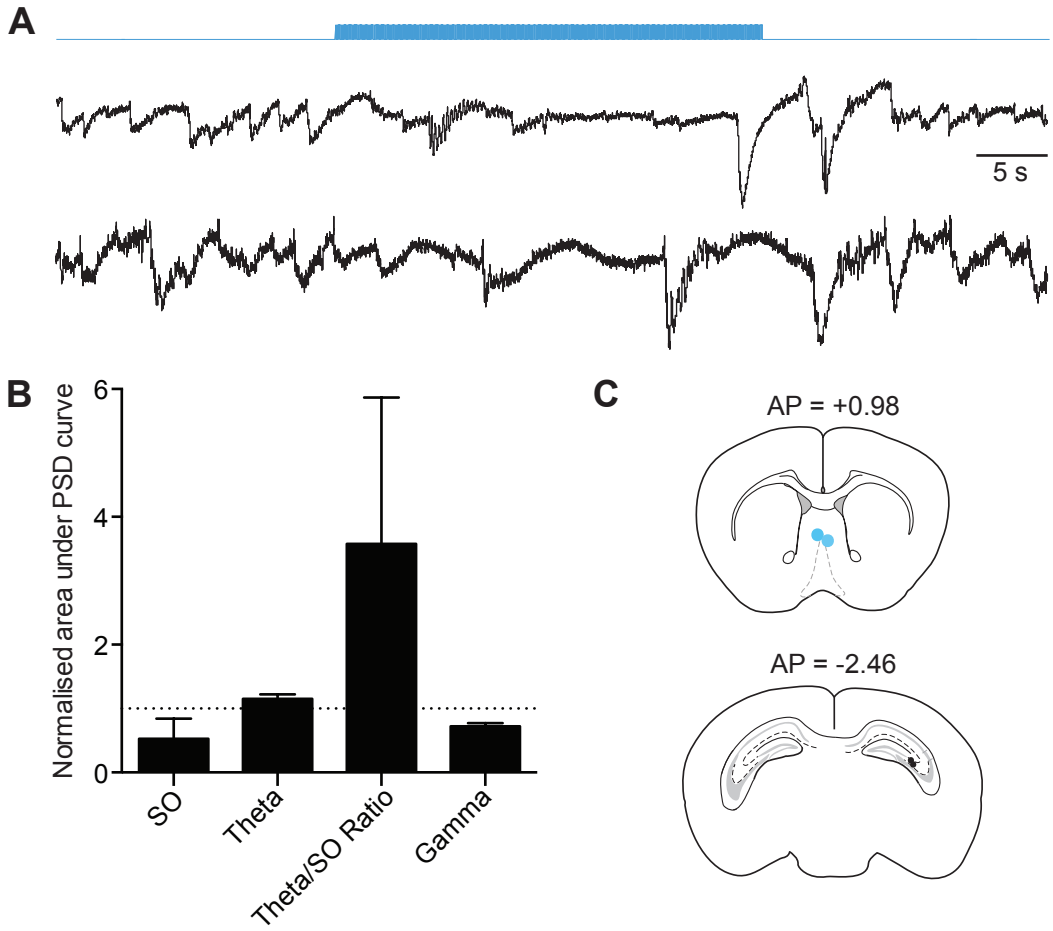


Figure 3.9: SO was also suppressed in freely-moving ChAT-Ai32 mice during light stimulation. (A) Two example traces of hippocampal LFP recordings during consummatory behaviour. Blue bar indicates light stimulation. **(B)** Area under the PSD curve for the frequencies of interest for the trace during 30 seconds of the test conditions (no light or 30 seconds of light stimulation) was calculated and normalised to the same area during baseline recording. Dotted line shows 1, indicating no change before and after test condition. n=2 from 2 mice. Error bars represent S.E.M. **(C)** The approximate locations of the tips of the fibre optic (blue) and electrode implant (black) for each mouse used in the recordings were verified post-recording. AP, Anterior-Posterior from Bregma

and DAPI (to visualise cell nuclei). Immunostaining revealed that ChAT-Ai40D mice showed selective GFP-positive staining in MS cholinergic neurons (Figure 3.10). 100% of ChAT-immunopositive cells were positive for ArchT-eGFP (GFP^+ChAT^+) in the MS while there was no ArchT-eGFP-immunopositive and non ChAT-immunopositive cells (GFP^+ChAT^-), demonstrating selective expression (97 GFP^+ChAT^+=100%, 0 $ChAT^+GFP^-$, 0 GFP^+ChAT^- in n=97 neurons in two mice).

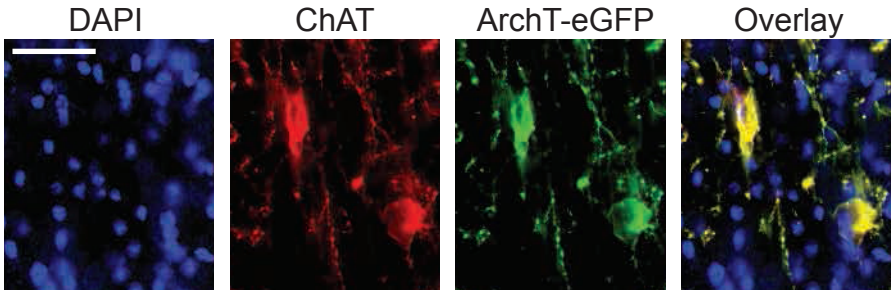


Figure 3.10: Selective expression of ArchT-eGFP in cholinergic neurons of ChAT-Ai40D mice. DAPI (blue), ChAT (red) and eGFP (green)-positive immunostaining in a coronal section of the medial septum of a ChAT-Ai40D mouse. Scale bar: 20 μm.

3.3.2 Light stimulation did not change MS MUA activity in ChAT-Ai40D mice

To test whether optogenetic silencing of cholinergic neurons could be detected in MUA activity, I performed MUA recordings in the MS of anaesthetised ChAT-Ai40D mice. Similar to recordings performed in ChAT-Ai32 mice, a stripped optical fibre bundled to a 1 MΩ tungsten recording electrode was lowered into the CA3. I tested a continuous 30-s period of silencing with 561 nm light. Spike frequency showed no significant differences before and during light stimulation (baseline spike frequency: 5.05±0.11spikes/s vs. 'light on' spike frequency: 5.01±0.10 spikes/s, two-tailed paired *t*-test: p=0.8; n=12 recordings repeated one to four times at each recording site from 4 mice; Figure 3.11). This observation was perhaps unsurprising since the firing rate of cholinergic neurons in

the MS is slow compared to the fast- and burst-firing non-cholinergic neurons (Serafin et al., 1996; Sotty et al., 2003; Simon et al., 2006). Therefore, it is possible that inactivation of cholinergic cells will not have a significant impact on MUA.

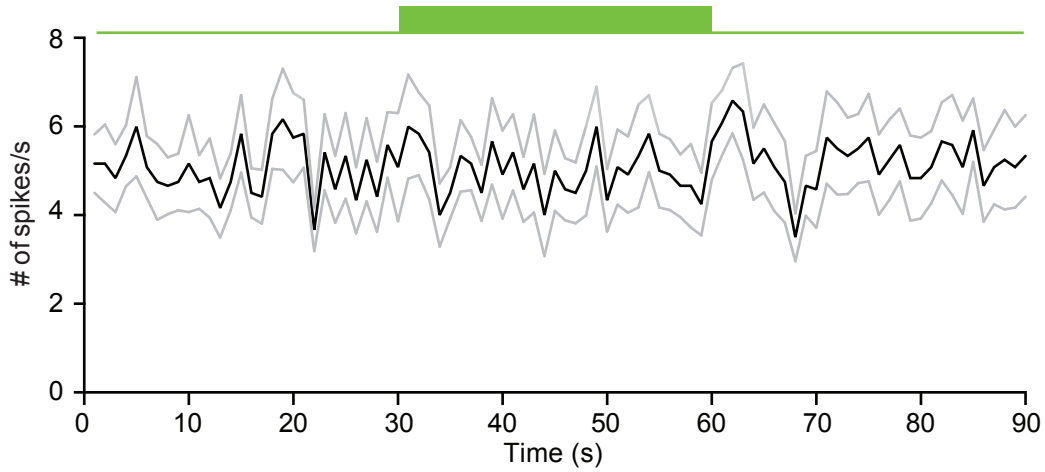


Figure 3.11: MUA activity in the MS of ChAT-Ai40D mice did not change with light stimulation. The mean frequency of spiking (n=12; black) and S.E.M. (grey). Green bar indicates light stimulation.

3.3.3 CA3 responses to cholinergic inactivation during SWR state

To investigate the role of septal cholinergic inactivation on hippocampal slow, theta and slow gamma oscillations, I lowered a 1MΩ tungsten electrode into the CA3 to record hippocampal LFP. Similar to experiments performed with ChAT-Ai32 mice, I recorded 60 s-long traces during SWR state with no light stimulation as a control. The final 30 s was compared to the first 30 s. I saw small fluctuations in the power of slow (SO; 0.5–2 Hz; 93.5±4%; n=13), theta (2–6 Hz; 113±18%; n=13) and slow gamma (20–40 Hz; 126±23%; n=13) oscillations and the theta/SO ratio (122±20%; n=13).

A second group of recordings with a 30 s-long baseline followed by 30 s-long continuous 561 nm light stimulation were then assessed and the differences between these two groups address the question of the effects of cholinergic inactivation on the frequencies of interest. As behavioural tasks can last longer than 30 seconds, a third group of recordings with a 60 s-long baseline followed by 60 s-long continuous 561 nm light stimulation were also made to ensure that cholinergic silencing can also be observed for this extended period.

Inactivation of cholinergic neurons in the MS during SWR state increased the power of SO. With 30 s-long stimulation, the power of SO increased to 163±16% (n=13) and with 60 s-long stimulation, SO power increased to 184±28% (n=14; Figure 3.12A). ANOVA to compare group means (i: no light, ii: 30 s light stimulation and iii: 60 s light stimulation) revealed significant differences in the means of the groups ($F(2,37)=6.033$, p=0.0054). Assuming local homogeneity, Gabriel post-hoc test revealed significant differences between the no light and 30 s light stimulation (p=0.047) and the no light and 60 s light stimulation (p=0.006) groups.

Theta and slow gamma oscillations did not show any significant differences between the groups. This is perhaps unsurprising as basal cholinergic tone during SWR state is low (Marighetto et al., 1993). It is however interesting to see that there was an increase in SO as ACh may play a role in modulating the power of SO during SWR state.

3.3.4 CA3 responses to cholinergic inactivation during theta-gamma state

ACh is known to play an important role in theta oscillations (Lee et al., 1994; Buzsáki, 2002; Li et al., 2007) and thus, I also investigated the impact of MS cholinergic activity during theta-gamma state by silencing them optogenetically. Inactivation of cholinergic neurons in the MS also increased the power of

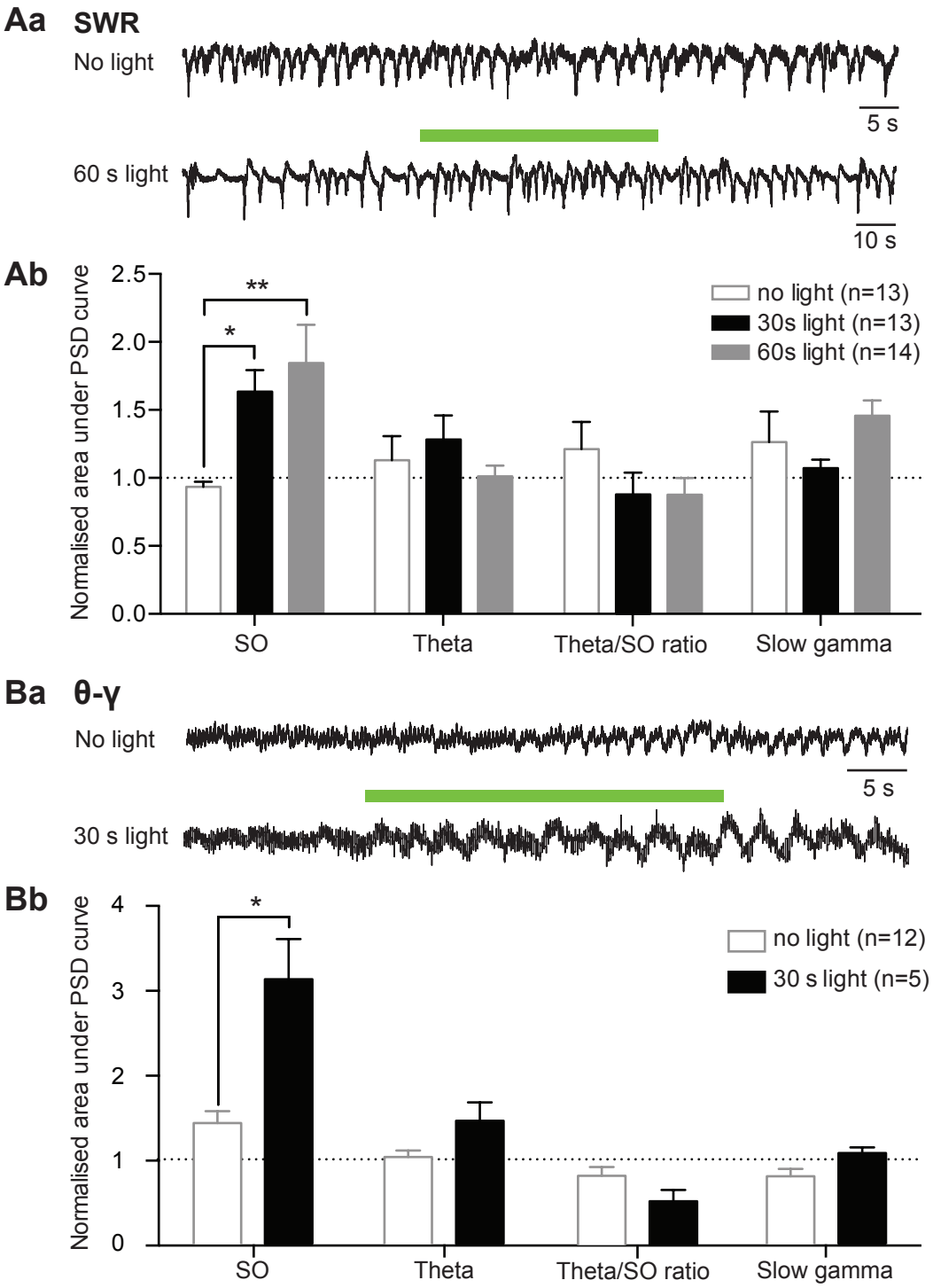

Aa
SWR
No light
5 s
60 s light
10 s
Ab
Normalised area under PSD curve
2.5
2.0
1.5
1.0
0.5
0.0
**
*
no light (n=13)
30s light (n=13)
60s light (n=14)
SO
Theta
Theta/SO ratio
Slow gamma
Ba
θ-γ
No light
5 s
30 s light
Bb
Normalised area under PSD curve
4
3
2
1
0
*
no light (n=12)
30 s light (n=5)
SO
Theta
Theta/SO ratio
Slow gamma

Figure 3.12: Cholinergic inactivation enhanced power of slow oscillations. **(Aa)** Example traces of hippocampal LFP recordings in ChAT-Ai40D mice during SWR state. Top: a baseline trace with no light stimulation. Bottom: an example trace with 60 s-long stimulation starting at 60 s. Green bar indicates time with continuous light stimulation. **(Ab)** Area under the PSD curve for the frequencies of interest for the trace during 30 s or 60 s of light stimulation was calculated and normalised to the same area during baseline recording. **(Ba)** Example traces of hippocampal LFP recordings during theta-gamma (θ-γ)state. Top: a baseline trace with no light stimulation. Bottom: an example trace with 30 s-long stimulation starting at 15 s. **(Bb)** Area under the PSD curve for the frequency of interest during 30 s of light stimulation was calculated and normalised to the same area during baseline recording. Dotted line show 1, indicating no change before and after light stimulation. *$p<0.05$; **$p<0.01$; ***$p<0.001$.

SO. With 30 s-long stimulation, the power of SO increased 312±47% (n=5) compared to 143±14% (n=12) with no light stimulation (Figure 3.12B). An unpaired *t* test to compare group means revealed a significant difference in the means of the groups (p=0.02; Welch's correction as equal SD was not assumed given the very different sample sizes). Theta and slow gamma showed no significant changes.

3.4 Dopaminergic activation

Not much is known regarding the effects of DA on hippocampal LFP, yet a number of studies have reported aberrant network activity in a number of neurological disorders involving the dopaminergic system such as schizophrenia (Haenschel and Linden, 2011; Gandal et al., 2012; Hunt et al., 2017). Thus, to investigate the effects of dopaminergic activation on hippocampal LFP, I used DAT-Ai32 mice which are offspring of DAT::IRES-Cre$^{+/+}$ mice crossed with the Ai32 line that expresses a Cre-dependent, eYFP-tagged ChR2 cassette. As a result, DAT-Ai32 mice express ChR2-eYFP in dopaminergic cell groups.

3.4.1 ChR2-eYFP expression in DAT-Ai32 mice is selective in dopaminergic neurons

To verify the level of transgene expression and selectivity, DAT-Ai32 mice were perfused and coronal sections of the VTA and hippocampus were made for immunohistochemistry. To visualise dopaminergic neurons, localisation of ChR2-eYFP and cell nuclei, the sections were stained for tyrosine hydroxylase (TH), YFP and DAPI, respectively. Immunostaining revealed selective eYFP-positive staining in VTA dopaminergic neurons (Figure 3.13). Over 80% of TH-immunopositive cells were positive for ChR2-eYFP (YFP^+TH^+) in the VTA while there was no YFP^+TH^- cells, demonstrating selective expression

(88 YFP^+TH^+ = 83%, 18 TH^+YFP^-, 0 YFP^+TH^- in n=106 neurons in two mice).

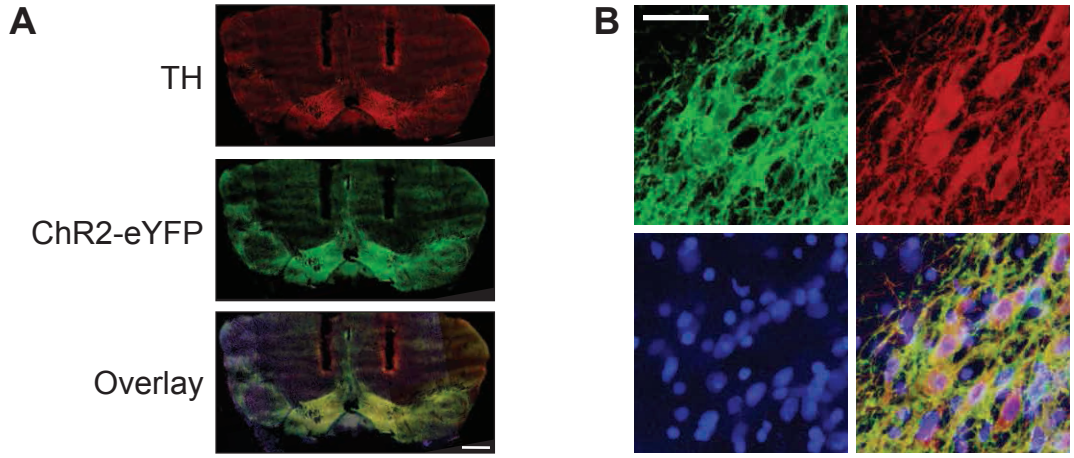


Figure 3.13: Selective expression of ChR2-eYFP in dopaminergic cells of DAT-Ai32 mice. (A) TH (top), eYFP-positive (middle) immunostaining in a coronal section of the VTA in a DAT-Ai32 mouse. Bottom: overlay of DAPI, TH and eYFP staining. Scale bar: 500 μm. **(B)** High magnification of the VTA showing DAPI (blue), TH (red) and eYFP (green)-positive immunostaining (and the overlay) in a coronal section of the VTA. Scale bar: 50 μm.

3.4.2 Dopaminergic activation decreased multi-unit activity in the VTA

To confirm that it was possible to activate putative dopaminergic neurons by illumination of the VTA, I first performed MUA recordings in the VTA of urethane-anaesthetised DAT-Ai32 mice. After recording a 30 s-long baseline of MUA, brief 50 ms-long pulses of 473 nm light at 10 Hz was applied to stimulate dopaminergic cells in the VTA for 30 s. Unexpectedly, MUA decreased (baseline spike frequency: 24.63±0.45 spikes/s vs. 'light on' spike frequency: 15.99±0.64 spikes/s, two-tailed paired *t*-test: $p<0.0001$; n=6 recordings repeated three times at each recording site from 2 mice; Figure 3.14).

To further analyse spikes in the MUA recordings, unsupervised spike clustering was performed using the Wave_clus programme (written in MatLab) developed

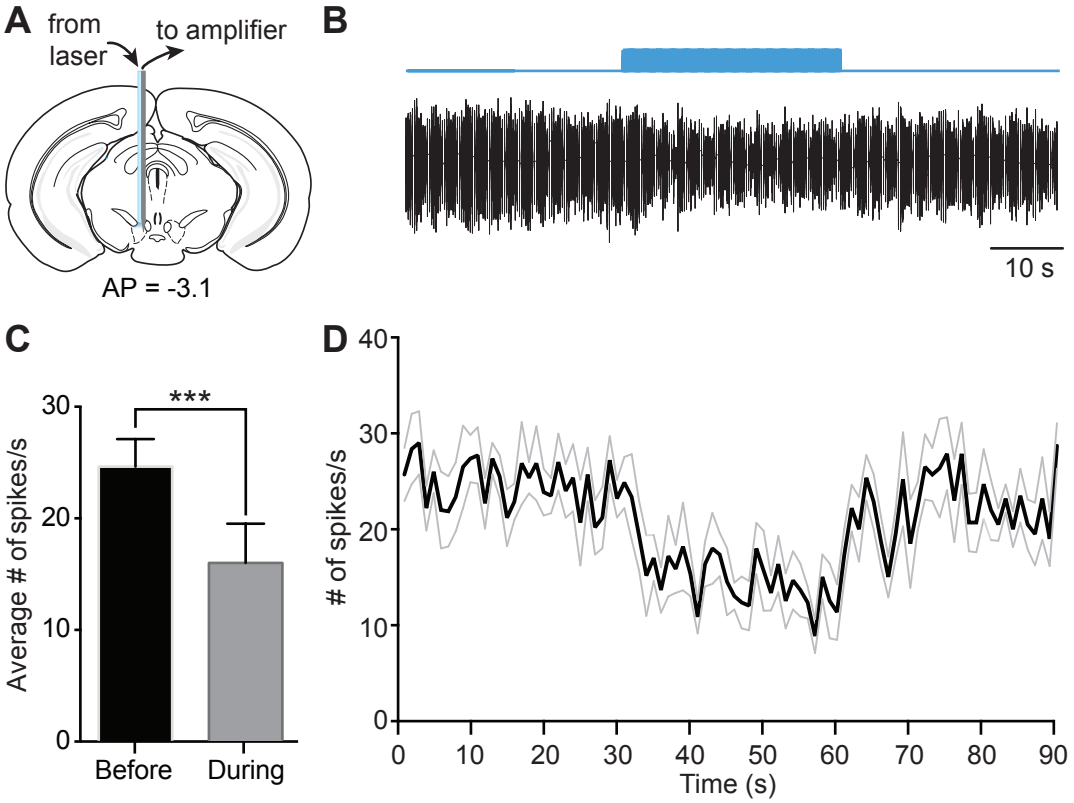


Figure 3.14: Multi-unit activity in the VTA of DATAi32 mice significantly decreased with light stimulation. (A) Schematic of the experimental set up. An optic fibre and electrode were lowered into the VTA to stimulate and record from VTA neurons. **(B)** Illumination of dopaminergic neurons in anaesthetised mice with blue laser light for 30 seconds (top) resulted in a reversible reduction in spontaneous spiking frequency. **(C)** The number of spikes per second was averaged over 30 seconds before and 30 s during light stimulation. (n=6; ***p<0.001) **(D)** The mean frequency of spiking for the duration of the recording (n=6; black). Grey lines indicate S.E.M.

and published by Quiroga et al. (2004). Based on various sets of spike features, as identified from wavelet transform, Wave_clus identified 3 spike clusters before light stimulation (arbitrary named '1', '2' and '3'). One of these clusters appear to be suppressed during stimulation (cluster '3'). After light stimulation, its spiking rate returned to a similar level compared to the spike rate during baseline (i.e. before the light stimulation; Figure 3.15).

3.4.3 CA3 LFP responses to VTA dopaminergic activation

To investigate the role of VTA dopaminergic activation on hippocampal slow, theta and slow gamma oscillations, I lowered a 1MΩ tungsten electrode into the CA3 to record hippocampal LFP. I recorded a 30 s-long baseline followed by a 30 s-long recording with 10 Hz, 25 mW, 473 nm light stimulation. During light stimulation, the power of fast (60-100 Hz) and slow (20-40 Hz) gamma oscillations significantly decreased 66±4% (one-sample *t*-test compared to 1: n=36 from 3 mice, $p<0.0001$) and 75±5%, respectively ($p<0.0001$; Figure 3.16). Power of SO and theta remained stable (SO: 96±4%, one-sample *t*-test compared to 1: n=36 from 3 mice, $p=0.37$; theta: 97±6%, $p=0.59$; theta/SO ratio: 104±6%, $p=0.51$).

3.5 Discussion

Using transgenic mice engineered to express optogenetics tools in cholinergic or dopaminergic cells, I have found that cholinergic activation suppressed SO and enhanced theta and gamma oscillations in the hippocampus of urethane-anaesthetised mice during both SWR and theta-gamma states. These effects peaked at 25 mW light intensity. The frequency of the ACh-facilitated hippocampal theta oscillations was independent of the frequency of light stimulation in the MS. However, the effects of the optogenetic cholinergic activation were de-

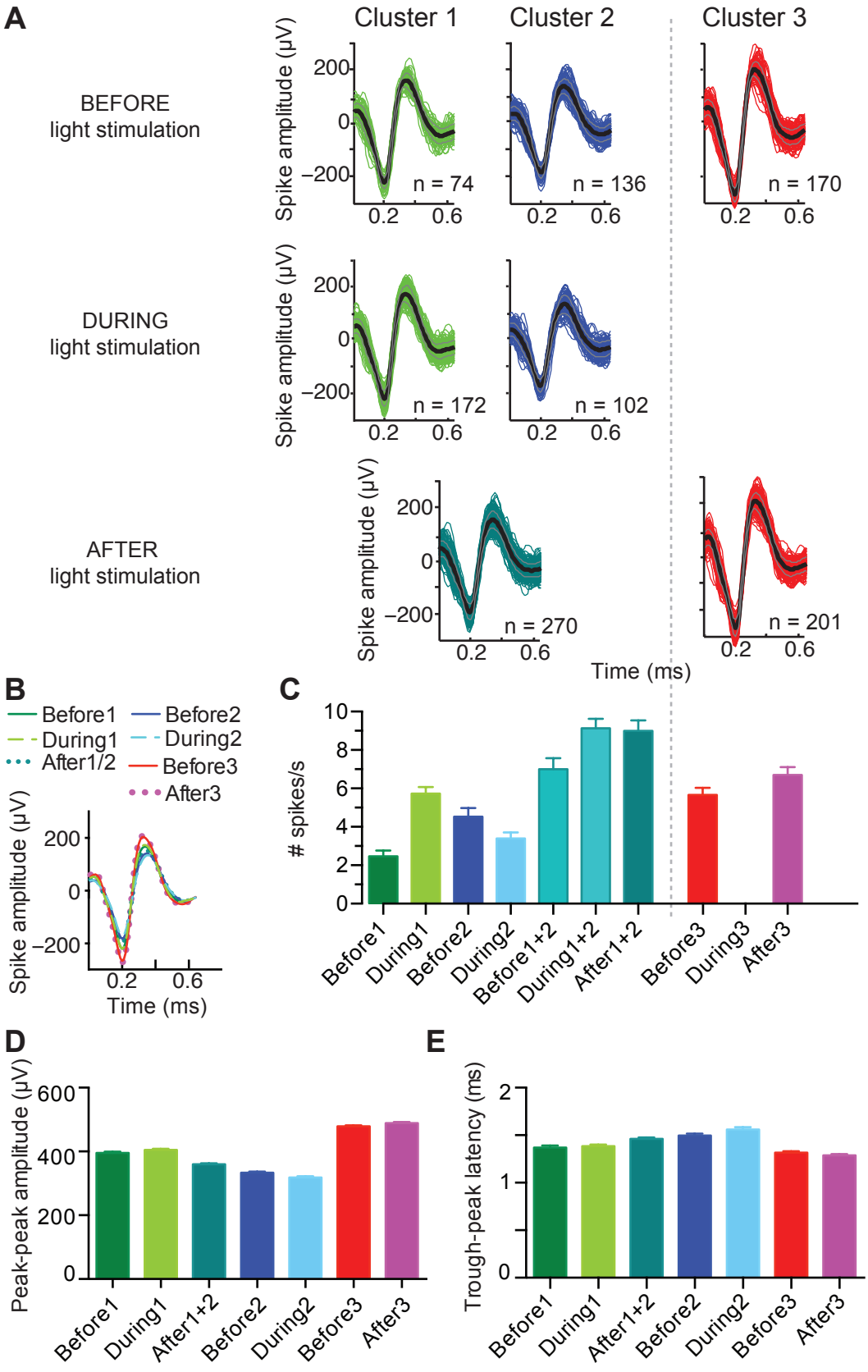

A
Cluster 1
Cluster 2
Cluster 3
BEFORE
light stimulation
DURING
light stimulation
AFTER
light stimulation
Spike amplitude (μV)
200
0
−200
n = 74
n = 136
n = 170
n = 172
n = 102
n = 270
n = 201
0.2
0.6
Time (ms)
B
Before1
During1
After1/2
Before2
During2
Before3
After3
Spike amplitude (μV)
Time (ms)
C
spikes/s
10
8
6
4
2
0
Before1
During1
Before2
During2
Before1+2
During1+2
After1+2
Before3
During3
After3
D
Peak-peak amplitude (μV)
600
400
200
0
E
Trough-peak latency (ms)
2
1
0

Figure 3.15: Dopaminergic activation appeared to suppress a cluster of spikes in the VTA. (A) Output of unsupervised spike clustering by Wave_clus identified 3 spike clusters (green, blue and red) before light stimulation (top). During light stimulation, the red cluster appeared to be suppressed (middle). After light stimulation, the red cluster continued spiking. **(B)** The average spike shape of each cluster from before, during and after stimulation were superimposed to demonstrate similar clusters from each recording segment. Before1=cluster 1 before light stimulation. **(C)** The average spike frequency of each cluster. Since Wave_clus was unable to separate clusters 1 and 2 after light stimulation, mean spike frequency for Before1 and Before2 were combined (Before1+2) and During1 and During2 spike frequencies were also combined (During1+2). **(D)** The average peak-to-peak amplitude of each cluster. **(E)** The average trough to peak latency of each cluster. Error bars indicate S.E.M.

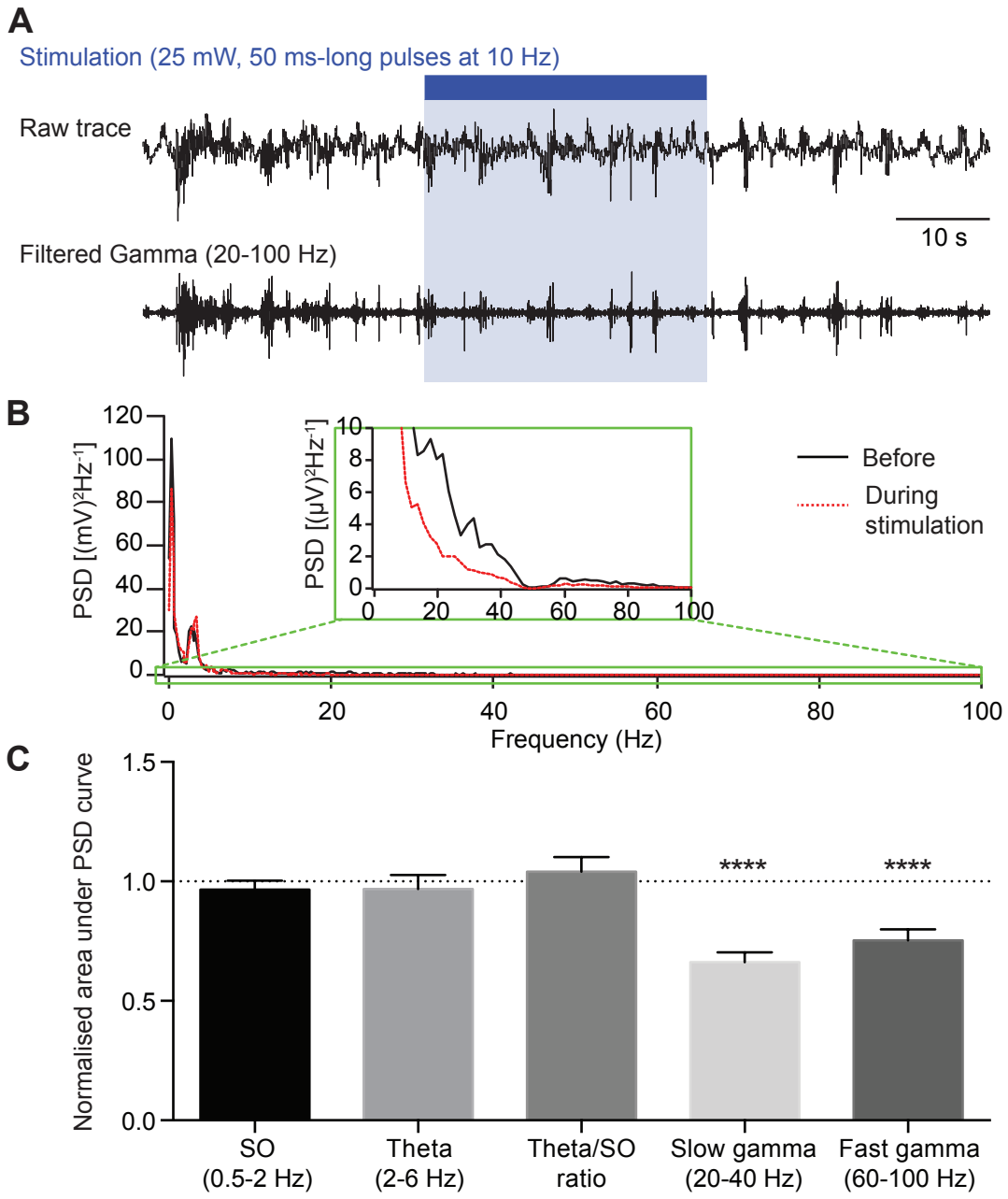


Figure 3.16: VTA dopaminergic stimulation suppressed gamma oscillations. **(A)** Example of hippocampal LFP displaying gamma oscillations (filtered 20-100 Hz in bottom panel) before, during and after 30 seconds of 473 nm 50 ms- long pulse stimulation. **(B)** The power spectral density (PSD) curve of an example trace before (black) and during stimulation (red). Inset depicts the frequency range of 0-100 Hz where during stimulation (red), the power of gamma oscillations decreased (20-100 Hz) during light stimulation. **(C)** Area under the PSD curve for the frequencies of interest for the trace during 30 seconds of light stimulation was calculated and normalised to the same area during baseline recording. Dotted line shows 1, indicating no change before and during light stimulation. ***$p<0.0001$.

pendent on muscarinic receptors as administration of the muscarinic antagonist scopolamine blocked these effects. Moreover, preliminary findings suggest that the cholinergic-suppression of SO can be replicated in freely-moving mice. In contrast, inactivation of cholinergic neurons enhanced hippocampal SO while activation of dopaminergic neurons suppressed hippocampal gamma oscillations in anaesthetised mice.

Furthermore, I verified that ChAT-Ai32, ChAT-Ai40D and DAT-Ai32 mice are reliable models for studying the cholinergic or dopaminergic system, respectively. Immunohistochemistry findings showed that expression of opsins was cell-type selective. Physiological effects of the opsins were also investigated using multi-unit activity recordings where, as expected, light stimulation of ChR2 in ChAT-Ai32 mice increased frequency of spikes. Conversely, stimulation of ArchT in ChAT-Ai40D mice revealed no change in spike frequency. This is unsurprising as septal cholinergic neurons display regular, low frequency tonic firing that could be overpowered by spontaneously active, burst firing septal non-cholinergic neurons (Serafin et al., 1996). Unexpectedly, stimulation of ChR2 in DAT-Ai32 mice decreased spike firing.

3.5.1 Effects of acetylcholine

3.5.1.1 Slow oscillations

Optogenetic cholinergic activation consistently suppressed SO in urethane-anaesthetised and freely-moving mice, an effect also observed by Vandecasteele et al. (2014). A recent *in vitro* study has also demonstrated that application of carbachol, a muscarinic agonist, stopped spontaneous SWRs (Zylla et al., 2013), supporting the findings in this thesis. Conversely, optogenetic cholinergic inactivation increased the power of SO. However, there appears to be a mismatch between the MUA and LFP data. Cholinergic inactivation did not appear to

have an effect on MUA activity in the MS, but in the hippocampus, SO power increased. MUA records the activity of multiple neurons around the electrode. In the MS, the proportion of cholinergic ($ChAT^+$) neurons is low (8.8%; Simon et al., 2006), quite sparsely distributed (40000/mm^3; Li et al., 2017), and, in comparison to the fast- and burst-spiking GABA (∼10–18 Hz) and glutamergic (∼8–14 Hz) neurons (Serafin et al., 1996; Sotty et al., 2003; Simon et al., 2006) also present in the MS, slow spiking (∼5 Hz). Thus, recording MUA in the MS may not be a sensitive enough technique to detect silencing of cholinergic neurons. More sensitive techniques such as single-unit recordings would be required. On the other hand, hippocampal LFP recordings measure population activity of neurons near the electrode and can thus characterise any direct or downstream effects cholinergic silencing may have on microcircuits. Nevertheless, these findings support the hypothesis that reduced cholinergic activity facilitates the initiation of SWRs by allowing effective spread of excitation in the recurrent CA3 network (Buzsáki et al., 1983). ACh has been shown to suppress the release of glutamate from presynaptic CA3 neurons and thus attenuate the spread of excitation required for SWR initiation (Hasselmo and Schnell, 1994; de Sevilla et al., 2002).

It is thought that the excitation required for SWR is regulated by axo-axonic cells. Inhibition of hippocampal CA3 axo-axonic cells, possibly mediated by bursting septal GABAergic neurons, in anaesthetised rats and freely moving mice was shown to abolish SWRs (Viney et al., 2013). Axo-axonic cells regulate spike generation by targeting the axon initial segment of pyramidal cells through GABAergic synapses and thereby suppressing the excitation required for SWRs. However, it is unclear why bursting activity occur in a specific subset of septal GABAergic neurons during reduced cholinergic tone, in the absence of theta oscillations. Nevertheless, together, these findings support the idea that muscarinic receptor activation suppresses SO and enhances theta oscillations.

3.5.1.2 Theta and gamma oscillations

My results provided direct evidence in support of the hypothesis that ACh has a passive role in the generation of hippocampal theta rhythm. While cholinergic activation enhances theta oscillations, inactivation of septal cholinergic neurons does not appear to impair theta power. However, it is very likely that only a proportion of cholinergic cells were silenced depending on the spread and intensity of light. Alternative experimental approaches such as chemogenetics (e.g. injection of Cre-dependent viral vectors containing inhibitory hM4Di receptors into the MS of ChAT-Cre mice) are required to confirm this observation.

Furthermore, the lack of entrainment by the stimulation frequency in ACh-facilitated theta oscillations adds further evidence that septal cholinergic neurons do not pace theta oscillations. Given the slow action of the second messenger-mediated mechanism of muscarinic receptors (Thiele, 2013), this is not surprising. It has been suggested that theta oscillations are instead paced by septal GABAergic neurons that exclusively synapse with hippocampal inhibitory interneurons (Freund and Antal, 1988; Dragoi et al., 1999; Hangya et al., 2009).

Moreover, it suggested that the cholinergic system also play an important role in gamma rhythms. Cholinergic activation via application of carbachol *in vitro* is sufficient to induce 40 Hz gamma frequency activity (Fisahn et al., 1998). Activation of kainate receptors (Hajos et al., 2004) and metabotropic glutamate receptors (Pálhalmi et al., 2004) also induces similar effects.

Interestingly, in Vandecasteele et al. (2014)'s study, they reported a differential effect for theta and gamma oscillations between urethane-anaesthetised and freely-moving mice where similar to my findings, during anaesthesia, theta oscillations were enhanced with cholinergic activation. However, the same stimulation exerted a less clear effect on theta rhythms in freely-moving, behaving mice. This difference could be attributed to distinct mechanisms responsible for

theta oscillations. Two types of theta rhythms have been reported (Vanderwolf, 1969; Buzsáki, 2002), one observed in anaesthetised animals where application of atropine eliminates theta (atropine-sensitive) and has been suggested to provide background sensory input-related drive to the hippocampus during high cholinergic tone. The other type of theta observed during movement is largely cholinergic independent (atropine-resistant) and is thought to be important for motor integration, but the type of receptor giving rise to this type of theta has never been conclusively identified. Thus, for the data presented in this thesis to be behaviourally-relevant, more recordings, especially those recorded during locomotion in freely-moving mice, are required.

3.5.1.3 Perspective

The principal findings in this chapter - enhancement of theta and gamma oscillations and suppression of SWR by ACh - further highlights the biphasic role of ACh in memory formation. During exploration, when a high concentration of ACh is present (Fadda et al., 2000), theta and gamma rhythms are enhanced, facilitating memory encoding by promoting neural communication and plasticity, the cellular process thought to underlie memory formation. During rest, concentration of ACh is low, SWRs are enhanced and thereby facilitate memory consolidation (Hasselmo and Mcgaughy, 2004).

This biphasic role of ACh has important implications for treatment of cognitive decline as one of the main treatments for AD are AChE inhibitors. AChE inhibitors prevent the breakdown of ACh and thereby, in theory, treat ACh deficiency observed in AD patients. While AChE inhibitors may be helpful in restoring the levels of ACh to physiological level in AD patients, a tonic increase in ACh level may not always be beneficial. A tonic increase in ACh suppresses SWRs required for memory consolidation which could impair memory formation. As such, this may account for the varying effects observed in AChE

inhibitor treatments (Forchetti, 2005; Birks, 2006; Martorana et al., 2010). To determine what, if any, behavioural phenotypes may be associated with this ACh suppression of SWRs, I intend to test behaving ChAT-Ai32 mice with selective activation at various phases of spatial memory tasks in the following chapters.

3.5.2 Effects of dopamine activation

3.5.2.1 Local VTA neurocircuitry

One of the more surprising results was that the activation of DA neurons induced a decrease in MUA in the VTA. Cluster analysis of the MUA data suggested that dopaminergic activation suppressed a cluster of spikes. This observation may be explained by a couple of interpretations.

Firstly, DA cells can be silent, firing single action potentials sporadically (tonic firing) or they can fire in bursts (phasic firing; Schultz et al., 1993; Schultz and Dickinson, 2000). How photostimulation affect this pattern of DA discharge is unclear. One possibility is that given the duration of the light pulses (50 ms), photostimulation could change DA firing pattern from phasic to tonic. While the shape of action potentials (AP) during tonic discharge remains more or less the same, the shape of APs in a burst vary with the first spike being sharper and larger than the second which is also sharper and ampler than the third AP in the burst and so on (Schiemann et al., 2012). The cluster that was suppressed during photostimulation appears to be large and sharp ('Before3': average AP amplitude=395±4.4 μV and average half-width=1.316±0.02 ms; Figure 3.15); this may represents a transition from burst to tonic AP discharge. In this case, the spike frequency would be expected to decrease.

Secondly, apart from receiving afferent inputs from neurons located in other brain structures such as the nucleus accumbens, local VTA neurons also regulate local GABA, glutamate and DA neurons (Nair-Roberts et al., 2008; Dobi et al., 2010; Chieng et al., 2011; Hnasko et al., 2012; Taylor et al., 2014; Morales and Margolis, 2017). *In vivo* and *in vitro* studies have revealed that dopaminergic tonic firing is driven by intrinsic pacemaker conductances. Local GABA neurons inhibit DA neurons via $GABA_A$ receptors (Johnson and North, 1992). As a result of this inhibition, the spontaneous firing rates of DA neurons are low. In contrast, a subset of GABA and glutamate neurons have been demonstrated to have fast spontaneous firing rates *ex vivo* (Margolis et al., 2006; Chieng et al., 2011; Hnasko et al., 2012). Thus, the identity of most of the recorded spikes at baseline could be VTA GABA and glutamate neurons. Upon optogenetic stimulation of DA neurons, activated DA neurons could regulate the activity of other VTA neurons. It has been suggested that in addition to DA release, midbrain DA neurons co-release GABA (Tritsch et al., 2012, 2014; Kim, Ganesan, Luo, Wu, Park, Huang, Chen and Ding, 2015; Ntamati and Lüscher, 2016) and, as such, activation of DA neurons could inhibit the activity of neighbouring neurons, thereby reducing the overall, summed MUA in the VTA.

Nevertheless, the findings from the cluster analysis may more support the first explanation as it is likely that all of the clusters identified are various APs from dopaminergic neurons. Compared to glutamate and GABA neurons, DA neurons are relatively large and can easily be recorded (Chieng et al., 2011; Morales and Margolis, 2017). The average half-width of these clusters are similar ('Before1'=1.369±0.02 ms, 'Before2'=1.496±0.02 ms, 'Before3'=1.316±0.02 ms, 'During1'=1.384±0.02 ms, 'During2'=1.559±0.03 ms, 'After1/2'=1.461±0.01 ms and 'After3'=1.287±0.01 ms; Figure 3.15E) and are comparable to the duration of VTA dopaminergic spikes published by other studies (Chieng et al., 2011; Berthet et al., 2014). The AP duration of GABAergic and glutamatergic neurons are shorter (Chieng et al., 2011; Hnasko et al., 2012; Morales and Margolis, 2017). Nevertheless, further investigation such as tetrode recordings and

single-unit recordings in the VTA would be needed to conclusively determine the local connectivity of DA neurons within the VTA and the role of this local VTA neurocircuitry in VTA function.

3.5.2.2 Hippocampal oscillations

My results suggest that activation of DA neurons reduces the power of baseline gamma oscillations. In the literature, there is conflicting data on the effects of DA on gamma oscillations due at least in part to methodological and conceptual variations (*in vitro* vs. *in vivo* recordings, knock-out models vs. acute treatments, general DA signalling vs. selective modulation of distinct subtypes of DA receptors). *In vivo* studies have found that application of acute dopaminergic agonists like *d*-amphetamine, apomorphine or methamphetamine to rodent cortex and hippocampus failed to induce any significant effects on baseline gamma activity (Ehrlichman et al., 2009; Pinault, 2008; Ma and Leung, 2000) or auditory evoked gamma power (Ehrlichman et al., 2009). On the other hand, *in vitro* studies in hippocampal slices have found that application of DA increased (Demiralp et al., 2007) or decreased (Ahveninen et al., 2000) the power and duration of stimulation-induced gamma oscillations. Likewise, the hyperdopaminergic dopamine-transporter knockout (DAT1-ko) mice showed increased hippocampal, but not cortical gamma power and duration (Dzirasa et al., 2006, 2009). These different methological variations modulate different sources of DA and types of receptors which could induce varying effects on hippocampal oscillations.

Early rodent behavioural studies using pharmacological manipulations of hippocampal D_1/D_5 dopamine receptors provided evidence that the hippocampal source of DA originated from the VTA and substantia nigra (Dahlström and Fuxe, 1964; Swanson, 1982; Lisman and Grace, 2005; Bethus et al., 2010). However, this innervation is sparse and the level of DA in the hippocampus is

much lower than in other regions like the striatum. VTA DA neurons exhibit bursting activity in response to reward or reward-prediction stimuli which in turn increase DA release in downstream areas including the hippocampus. Three recent publications have suggested that an additional source of hippocampal DA arises from noradrenergic neurons of which the locus coeruleus may also underpin memory associated signalling in the hippocampus (Smith and Greene, 2012; Kempadoo et al., 2016; Takeuchi et al., 2016). Further investigations are needed to identify the pathways involved and discern the relationship between DA and hippocampal gamma activity.

The results presented in this chapter also suggest that dopaminergic activation *in vivo* did not appear to have an effect on the other oscillatory frequencies. In the literature, how DA affects SWR activity is unclear. Much like gamma oscillations, conflicting data have been reported. The effects of DA on hippocampal pyramidal cell excitability (a network state that promotes SWRs) are variable, with some studies reporting a decrease in excitability, e.g. application of DA to CA1 pyramidal cells hyperpolarises the membrane potential and increases the spike afterhyperpolarisation (Benardo and Prince, 1982), while others found that DA induced an increase in excitability, suppressing afterhyperpolarisation (Pedarzani and Storm, 1995).

Theta activity appears to decrease with depletion of DA in the hippocampus (Nakagawa et al., 2000) while injection of DA or non selective DA agonist apomorphine into the medial septum and diagonal band (MSDB) increased hippocampal theta activity in anaesthetised rats (Miura et al., 1987). The major source of DA in the MSDB is thought to be derived from the locus coeruleus and medulla oblongata. VTA dopaminergic neurons are thought to innervate neurons in the lateral septal nucleus (Lindvall and Stenevi, 1978; Swanson and Cowan, 1979). This could explain why activation of VTA DA neurons had no effect on theta activity. However, voltammetry in the hippocampus and further experiments using viral injections to more locally target VTA neurons is

required to confirm this hypothesis.

3.5.2.3 Perspective

The results in this chapter provided some new and interesting clues to the effects of DA on the VTA local neurocircuitry and hippocampal gamma oscillations. The mechanisms and receptors that underpin these effects remain to be elucidated.

VTA DA neurons have been shown to be excited during rewarding stimuli and aversive stimuli or cues that predict an aversive outcome can also excite or inhibit VTA DA neurons (Schultz et al., 1997). Furthermore, a recent study revealed that optogenetic activation of DAT-expressing input originating from the VTA to the dorsal hippocampus during learning improved recall in a difficult spatial learning task (McNamara et al., 2014). However, recent findings have questioned the influence of VTA dopaminergic input in the hippocampus given their sparse innervation (Lisman and Grace, 2005; McNamara et al., 2014; Rosen et al., 2015). DA release from noradrenergic fibres from the locus coeruleus (LC-TH$^+$) have recently been proposed to underpin DA associated modulation of hippocampal memory (Smith and Greene, 2012; McNamara and Dupret, 2017). LC-TH$^+$ more densely innervate the rodent hippocampus compared to DA fibres from the VTA (Smith and Greene, 2012; Kempadoo et al., 2016). In addition, activation of LC-TH$^+$ enhanced learning of various spatial learning tasks (Takeuchi et al., 2016; Kempadoo et al., 2016). Given the importance of DA signalling in memory formation and its involvement in several devastating disorders (e.g. schizophrenia, Parkinson's Disease), understanding how DA activity is controlled and their resulting activity in the hippocampus is critical.

Chapter 4

Effects of cholinergic modulation on working memory

4.1 Introduction

Working memory (WM) is a form of short-term memory in which the information is represented, maintained and updated for a short period of time (Dudchenko, 2004; Cowan, 2008; Aben et al., 2012). WM is modulated by cholinergic activity (Fadda et al., 1996); generally, interventions that inhibit cholinergic signalling impair WM performance while agents that enhance cholinergic signalling improve performance (for review, see Deiana et al., 2011). However, the results have not been uniform.

Differences in experimental set-up, species or task structure may explain differences in the effects of ACh on cognitive performance (Shen et al., 1996; Pizzo et al., 2002). Although intrahippocampal infusions of muscarinic antagonists (Brito et al., 1983; Izquierdo et al., 1992; Kim and Levin, 1996; Carli et al., 1997) or nicotinic antagonists (Ohno et al., 1993; Felix and Levin, 1997; Levin et al., 2002) impair hippocampal-dependent memory, studies involving the selective lesions of cholinergic projections to the hippocampus are more difficult to interpret. Their effects are highly dependent on the spread of the lesions; widespread lesions have been shown to impair spatial memory (Nilsson et al., 1992; Berger-Sweeney et al., 1994; Leanza et al., 1995), but more selective lesions have failed to induce any deficits in spatial learning in the water maze or spatial WM in the radial arm maze (Torres et al., 1994; Baxter and Gallagher, 1996; Baxter et al., 1996; McMahan et al., 1997; Vuckovich et al., 2004). Moreover, the effects of ACh are highly dependent on task demand. Studies have suggested that cholinergic antagonists impair spatial WM, but not spatial reference memory (Eckerman et al., 1980; Wirsching et al., 1984; Buresová et al., 1986; Ohno et al., 1993). Furthermore, WM can be split into 3 stages: encoding, maintenance, and retrieval (Marr, 1971; Gibbs and Ng, 1979; Buzsáki, 1989). In particular, cholinergic modulation has been implicated in the encoding of new information; cholinergic stimulation is thought to enhance neural activity associated with encoding while reduced

activity is linked to retrieval (Hasselmo and Mcgaughy, 2004; Kukolja et al., 2009). Together, these findings indicate that hippocampal ACh participates in spatial memory functions. However, septo-hippocampal cholinergic neurons may not be necessary for certain aspects of hippocampal-dependent memory, as performance on some spatial learning tasks survives the loss of these neurons.

To date, much of our knowledge on cholinergic modulation of learning and memory processes has been derived from genetic knockout models, ablation of brain regions or cell groups and administration of receptor agonists or blockers (Stevens, 1981; Riekkinen et al., 1991; Levin and Rose, 1995; Maviel and Durkin, 2003; Ohno et al., 2004; Seeger et al., 2004; Okada et al., 2015). These techniques lack temporal precision and reveal few clues to the context of neuromodulator action. The development of optogenetic approaches has allowed the opportunity to directly address questions regarding what stages of various kinds of memory each neuromodulator is involved in, by giving the possibility to reversibly activate or inactivate cells of interest with milliseconds precision in normally behaving animals (Boyden et al., 2005; Han et al., 2011).

4.1.1 Aim

Previous studies have reported mixed results while investigating the relationship between the cholinergic system and WM (Deiana et al., 2011). Their findings varied depending on task demands and experimental design. Few studies have used optogenetics to directly interrogate the effects of the cholinergic system on behavioural learning and memory tasks. Using an optogenetic approach, I aimed to determine the causal role of ACh modulation on hippocampal activity on two WM tasks: the spontaneous T-maze alternation task and the spontaneous location recognition (SLR) task. To further understand how ACh affects WM, I induced or prevented ACh release during different stages of WM. The tasks were divided into 3 stages: Phase 1 (i.e. acquisition of the WM

task); delay (i.e. a short maintenance period); and Phase 2 (i.e. retrieval of the previously encoded memory). Light was delivered during both phases, or either Phase 1 or Phase 2. Since these tasks are spontaneous, each mouse was repeatedly tested for each delay and light stimulation condition. Mice were also food-deprived to encourage exploration (Adlerstein and Fehrer, 1955; Hughes, 1965; Hughes and Swanberg, 1970).

Overall, given the importance of the cholinergic system in learning and memory processes, I hypothesised that cholinergic activation would enhance memory processes, particularly during the acquisition of the task. Conversely, I hypothesised that cholinergic inactivation would lead to learning deficits.

4.2 Spontaneous alternation T-maze task

The spontaneous alternation T-maze task is a task used to assess hippocampal-dependent spatial short-term memory (Deacon and Rawlins, 2006). It is based on the rodents' strong preference to explore a new and different environment. Mice were food-deprived and first habituated to handling before beginning the task. In this task, mice were first placed facing outwards in the start arm of the T-maze and allowed to enter either the left or right arm. A barrier was placed to contain them in their chosen arm for 30 seconds for exploration. After exploration, mice were removed from the arm and the barrier was removed. The mice was placed back at the start arm (facing outwards) immediately or after a delay period spent in their home cage. The subsequent arm choice was recorded (Figure 4.1A). Mice tend to enter the arm not visited before, indicating memory of the previous choice (Zhang et al., 2013). Each mouse received at most two trials per day, a total of 10 trials per condition. Mice typically spontaneouly alternate in 70-80% of trials (Deacon and Rawlins, 2006).

A previous line of ChAT-ChR2-EYFP mice (B6.Cg-Tg(Chat-COP4*H134R/EYFP, Slc18a3)6Gfng/J; Jackson stock #014546) displayed abnormal behaviour, showing dysfunction in spatial memory (Kolisnyk et al., 2013). Therefore, to ensure that the ChAT-Cre mouse line I use display normal behaviour, I first compared the performance of male heterozygous ChAT-Cre mice (Jackson Laboratories, stock #006410, n=9) to control mice (negative littermates or WT C57BL/6J mice bought from Jackson Laboratories, n=10) on the spontaneous alternation T-maze task (Figure 4.1) with delay periods of 0, 1, 2, 5 and 10 minutes.

Both groups showed equivalent levels of spontaneous alternation, indicating that there is no obvious genotype difference. On average, with delay periods of 0, 1 and 2 minutes, WT mice were spontaneously alternating in 76±23%, 79±14% and 70±17% of trials, respectively. In comparison, heterozygous ChAT-Cre (HET) mice alternated in 68±23%, 86±16% and 84±13%. A second set of trials was carried out with delay periods of 0, 5 and 10 minutes. WT mice on average alternated in 74±7%, 57±6% and 60±5% of trials, respectively while HET mice alternated in 71±5%, 72±5% and 68±5% of trials (two-way ANOVA: main effect of delay: $F(4,104)=3.89$, $p=0.0055$ but no main effect of genotype: $F(1,104)=2.74$, $p=0.1011$ nor effect of interaction between genotype and delay: $F(4,104)=1.61$, $p=0.18$).

4.2.1 Cholinergic activation did not have a conclusive effect on the spontaneous alternation T-maze task

A number of studies have implicated a strong involvement of ACh in spatial WM processing. Electrolytic abolition of the MS was suggested to impair performance in the T-maze (Kitabatake et al., 2003); mAChR antagonists impair spontaneous alternation (Grauer and Kapon, 1996; von Linstow Roloff et al., 2007; Hodges et al., 2009), while mAChR agonists enhance task performance

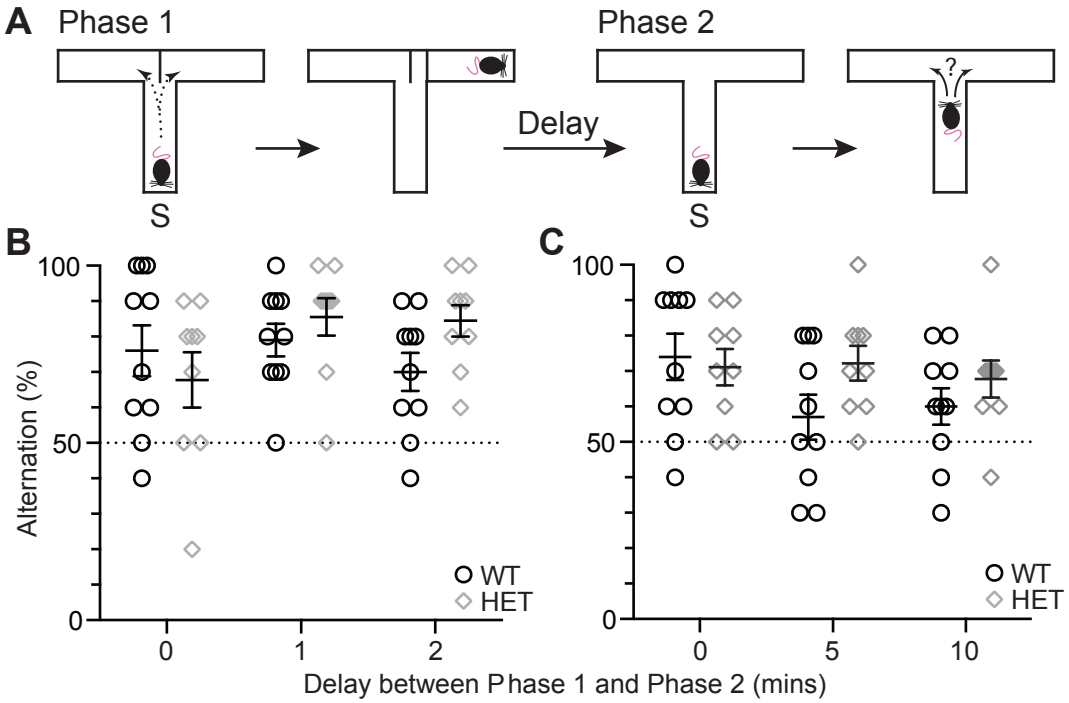


Figure 4.1: Performance on the hippocampus-dependent short-term memory spontaneous alternation T-maze task did not differ significantly between wild-type and heterozygous ChAT-Cre mice. (A) Schematic of the spontaneous alternation T-maze task. Mice were placed in the start arm (S) and allowed to choose an arm of their choice. Once they entered an arm, a barrier was lowered to allow mice to explore their chosen arm for 30 seconds (Phase 1). Mice were then removed and put into the home cage for the length of the delay period. After the delay period had elapsed, mice were placed back in the start arm and their arm choice was recorded (Phase 2). If the mice chose the novel arm, this was counted as a spontaneous alternation. **(B)** The average rate of spontaneous alternation in wild-type (WT; n=10) and heterozygous ChAT-Cre (HET; n=9) mice with 0, 1 and 2 mins delay between Phase 1 and Phase 2. **(C)** Spontaneous alternation in WT and HET mice with 0, 5 and 10 mins delay. Each symbol indicates one mouse. Broken lines represent chance performance of 50%. Error bars represent S.E.M.

(Shannon et al., 1990). In addition, nAChR agonists and AChE inhibitors were shown to reverse impairments induced by scopolamine or had no effect on the spontaneous alternation T-maze task (Ordy et al., 1988; Riekkinen et al., 1991; Bejar et al., 1999; Taylor et al., 2005). Differences in dose, strain or species, task structure, or level of training may explain variations in the effects of ACh modulators on cognitive performance. I intended to contribute to this field by dissecting the effects of cholinergic activation during the different phases of two WM tasks by using the relatively new technique, optogenetics, which offers cell-type specific modulation and temporal precision.

To determine the effects of cholinergic activation on different phases of spatial WM, I implanted 11 ChAT-Ai32 mice with an optic fibre cannula in the MS. Pulses of 473 nm light (50 ms-long pulses at 10 Hz frequency at 25 mW) were applied to the MS to activate cholinergic neurons. I tested three delay periods (0, 5 mins and 30 mins) and four light conditions ('no light', light on for 'both phases', light on for 'Phase 1 only' and light on for 'Phase 2 only'). Each mouse performed 10 trials for each delay period and each light condition (i.e. a total of 120 trials) to allow for repeated measures comparison.

At 0 min delay, mice performing the task under the 4 light conditions showed similar levels of spontaneous alternation ('no light'=57±7%, 'both phases'= 55±5%, 'Phase 1 only'=58±8%, 'Phase 2 only'=61±7%). At 5 mins delay, mice also showed similar levels of spontaneous alternation for the 4 light conditions ('no light'=46±8%, 'both phases'=56±6%, 'Phase 1 only'=60±7%, 'Phase 2 only'=60±9%). At 30 mins delay, spontaneous alternation for each groups were'no light'=29±6%, 'both phases'=51±7%, 'Phase 1 only'=43±7%, 'Phase 2 only'=48±7%; Figure 4.2). Repeated measures two-way ANOVA (within-subject factors of delay and light) revealed that there was a significant main effect of light: $F(3,30) = 5.212$, $p=0.005$ and delay: $F(2,20) = 14.324$, $p<0.001$ but no significant effect of the interaction between light and delay: $F(6,60) = 1.592$, $p=0.165$. However, importantly, compared to the expected

80% spontaneous alternation, the average spontaneous rate (even with no delay) was quite low and as such, it is difficult to draw any definitive conclusion from this experiment.

To more closely examine why the alternation rate was so low compared to other published studies (Deacon and Rawlins, 2006), I analysed the data more closely and found that most of the mice had an arm preference and over the period of 120 trials, alternation rate decreased, likely due to loss of motivation (Figure 4.3). This suggests that it may not have been ideal to require each mouse to perform 120 repetitions of the task. The arm preference displayed in these mice appear to be stronger than their tendency to alternate and as a result, the results from this experiment are hard to interpret. The increase in alternation rates between the 'no light' and 'both phases' light conditions with 30 mins delay appear to suggest that light stimulation may remove this arm preference.

4.2.2 Cholinergic inactivation during the spontaneous alternation T-maze did not appear to have an effect on performance

I hypothesised that the poor spontaneous alternation rate observed in ChAT-Ai32 mice was likely due to the large number of trials each mouse was required to perform. Thus, to alleviate this issue, when designing the experiments to test the effects of cholinergic inactivation on short-term hippocampus-dependent memory, I split the cohort of 12 ChAT-Ai40D mice evenly into 4 groups of 3 mice to test 4 light conditions ('no light', light on for 'both phases', light on for 'Phase 1 only' and light on for 'Phase 2 only') and two delay periods (0 and 10 mins). In total, each mouse performed 20 trials.

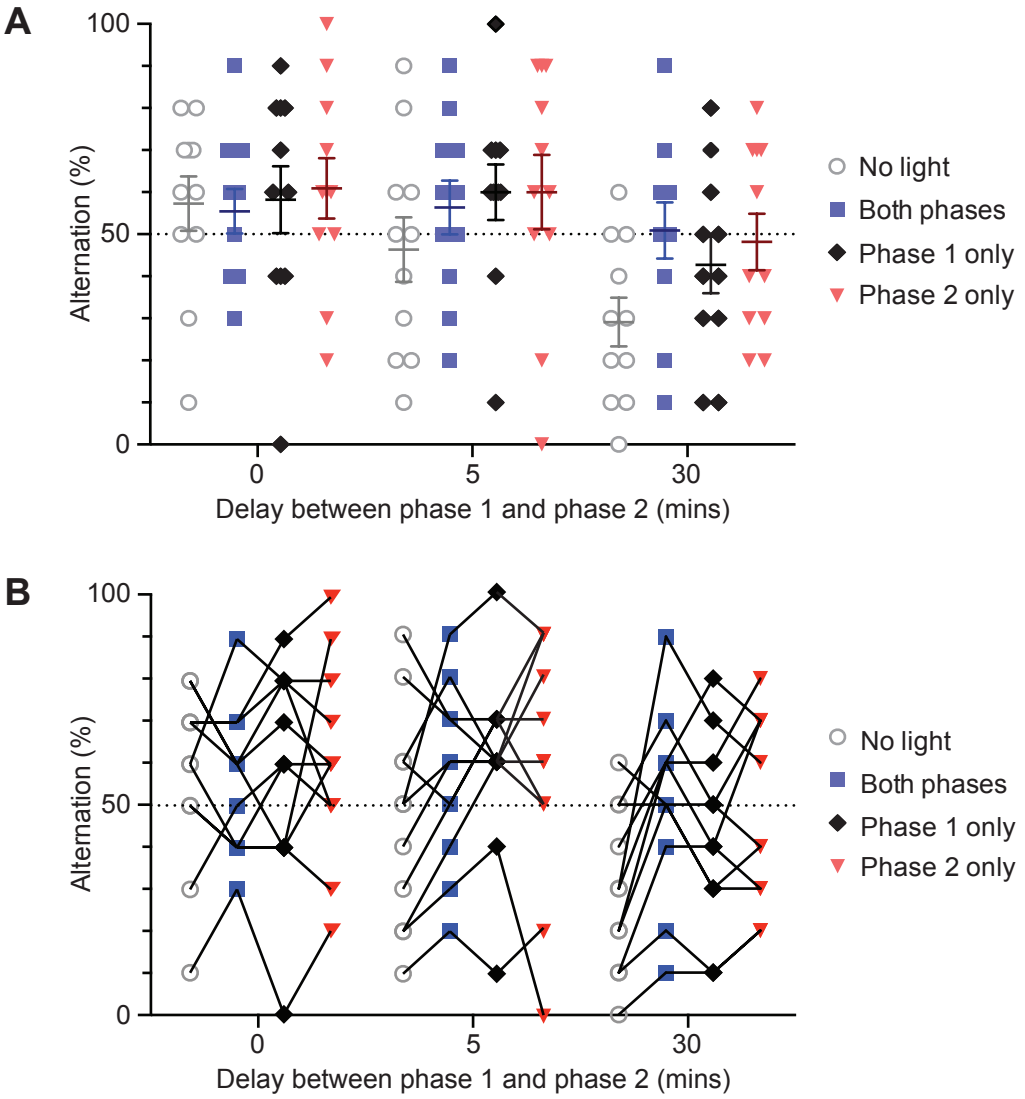


Figure 4.2: Due to the low rate of spontaneous alternation and large variability in the data, results of this experiment was inconclusive. (A) Spontaneous alternation rate in ChAT-Ai32 (n=11) mice with 0, 5 and 30 mins delay between Phase 1 and Phase 2 under 4 different light conditions: no light (white circles); light on for both phases (blue squares); light on for Phase 1 only (black diamonds); light on for Phase 2 only (red triangles). Each symbol indicates one mouse. Error bars represent S.E.M. **(B)** Spontaneous alternation rate for the 4 light conditions following each mouse during the T-maze alternation task with 0, 5 and 30 mins delay. Broken lines indicate chance performance of 50%.

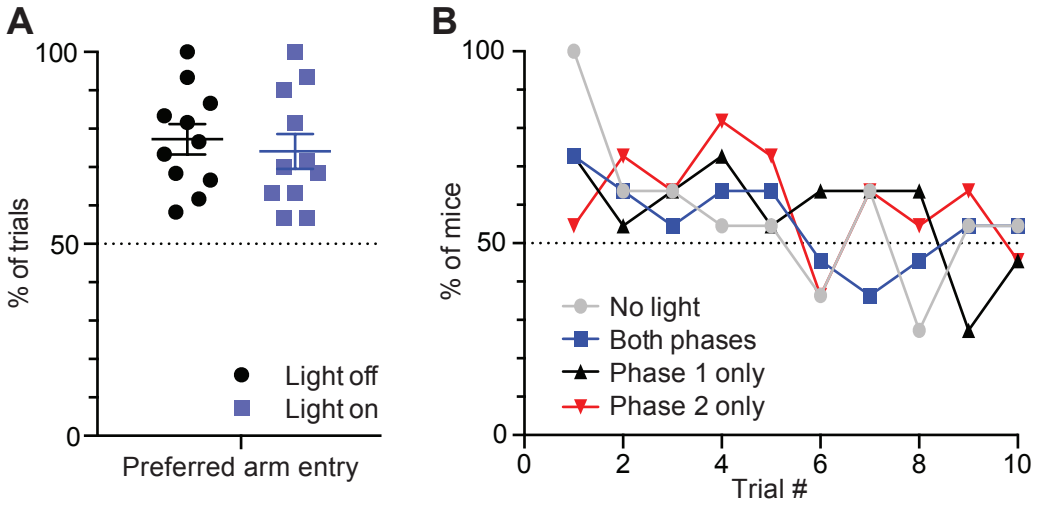


Figure 4.3: Most mice have an arm preference and over the number of trials, alternation rate decreases. (A) The percentage of trials in which ChAT-Ai32 mice (n=11) entered their preferred arm during Phase 1 of the T-maze task. Each symbol represents one mouse. There is no significant difference between light on (blue) and light off (black) conditions. Error bars represent S.E.M. **(B)** Percentage of ChAT-Ai32 mice that alternated over the course of 10 no delay trials under 4 different light conditions: no light (grey); light on for both phases (blue); light on for Phase 1 only (black); light on for Phase 2 only (red) average across the mice cohort. Broken lines represent chance performance of 50%.

On average, the spontaneous alternation rate was higher but there appear to be no significant effect of cholinergic inactivation on the performance of spontaneous T-maze alternation task. At 0 min delay, all four groups showed equivalent levels of spontaneous alternation ('no light'=87±7%, n=3; 'both phases'=77±3%, n=3; 'Phase 1 only'=77±7%, n=3; 'Phase 2 only'=93±3%, n=3; one-way ANOVA: $F(3,8)=2.4$, p=0.143). At 10 mins delay, mice also showed similar levels of spontaneous alternation for the 4 light conditions ('no light'=70±15%, 'both phases'=67±9%, 'Phase 1 only'=70±6%, 'Phase 2 only'=70%; one-way ANOVA: $F(3,8)=0.032$, p=0.992). This suggests that cholinergic inactivation may not have an effect on short-term hippocampus-dependent memory. This is consistent with previous findings where light stimulation in the MS of ChAT-Ai40D mice do not appear to have a strong effect on anaesthetised *in vivo* recordings (Section 3.3). Nevertheless, the sample sizes are very small and as such, may not have enough power to detect any effect cholinergic inactivation may have had (Marsh et al., 1988).

With a sample size of 12 mice, I would have had a 90% chance of detecting an effect size of 1.4 with a significance level of 0.05. However, the effect sizes observed in the T-maze alternation experiment with ChAT-Ai40D mice were smaller (Figure 4.4). With 0 min delay, the one-way ANOVA effect size was 0.752, which would require a sample size of 8 mice in each treatment group to detect an effect of light with 90% power and a significance level of 0.05. With 10 mins delay, the one-way ANOVA effect size was 0.25 and the power analysis revealed that a total sample size of 228 mice would be needed. Therefore, to detect any significant effects of cholinergic inactivation in the spontaneous alternation T-maze task with 0 min delay, a total sample of at least 32 mice would be required. A much larger sample size (n=228 mice) would be required to detect an effect of light for the T-maze task with 10 mins delay, which may not be feasible and it would be wise to abandon this delay condition in future experiments.

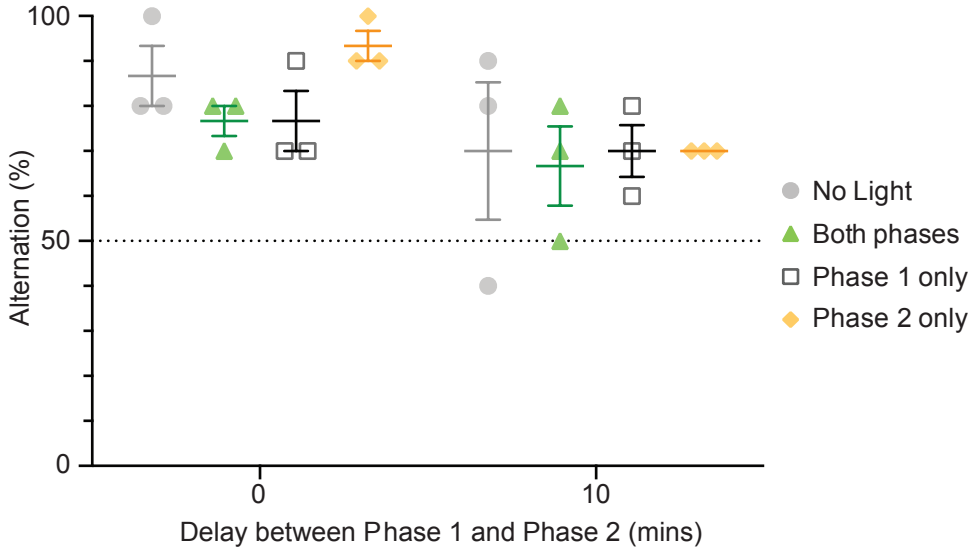


Figure 4.4: Inactivation of the cholinergic system does not appear to affect performance on the T-maze alternation task. Spontaneous alternation rate in ChAT-Ai40 mice (n=3 in each group) with 0 and 10 mins delay between Phase 1 and Phase 2 under 4 different light conditions: no light (grey); light on for both phases (green); light on for Phase 1 only (white); light on for Phase 2 only (yellow). Each symbol indicates one mouse. Broken line represents chance performance of 50%. Error bars represent S.E.M.

4.3 Spontaneous location recognition task

As an alternative task to investigate the effects of cholinergic modulation on WM, I also carried out the SLR task (previously described by Ennaceur et al., 1997) with ChAT-Ai32 and ChAT-Ai40D mice. The SLR task is used to assess a rodent's ability to discriminate whether an object it has previously encountered in the same test arena has moved. Much like the spontaneous alternation T-maze, it exploits the strong novelty preference displayed by rodents.

Mice were food-deprived and first habituated to handling and were allowed to explore the empty test arena freely prior to the start of the testing period. The testing arena was placed in the centre of a testing room that had distinct distal and proximal spatial cues. In this task, each trial consisted of two phases (Figure 4.5A). During Phase 1, each mouse started in the centre of the arena and was presented with two identical objects. It was allowed to explore the arena

for 3 mins, before it was removed and placed back in their home cage. After a delay period, each mouse was returned to the arena for Phase 2. In Phase 2, the mouse was presented with the same two identical objects previously used during Phase 1 but one in its previous, familiar location (i.e. non-displaced object) and the other placed in a novel location (i.e. displaced object). It was allowed to explore for 3 mins. The time each mouse spent exploring each object (defined as directing its nose to the object at a distance of 2 cm or less) was recorded and the total time spent exploring the objects in Phase 1 and 2 (e1 and e2), the proportion of time spent exploring object 1 during Phase 1 (p1) and the discrimination ratio (d2 = the difference between the proportion of time spent exploring the displaced object during Phase 2 and the proportion of time spent exploring the non-displaced object during Phase 2; Table 2.1) were calculated. All mice were tested concurrently, with a minimum interval of 48 hours between each trial. The mice were presented with new (to the mouse) objects for each trial.

I first performed this task with C57BL/6J to determine a baseline performance for various delay periods (Figure 4.5). I tested delay periods of 5 (n=4), 30 (n=5), 45 (n=8), 60 (n=6) and 90 (n=4) mins. As this was a spontaneous task, each mouse was tested in multiple delay periods. For all of the test conditions, mice spent on average a larger proportion of time exploring the displaced object as indicated by the positive d2 values, suggesting that mice seem to prefer exploring the object at a new location during Phase 2. However, only d2 values from experiments with delay periods of 45 and 60 mins were significant from chance exploration (i.e. d2=0; one sample *t*-test: 45 mins: $t(7)=5.354$, p=0.001, 60 mins: $t(5)=3.971$, p=0.0106).

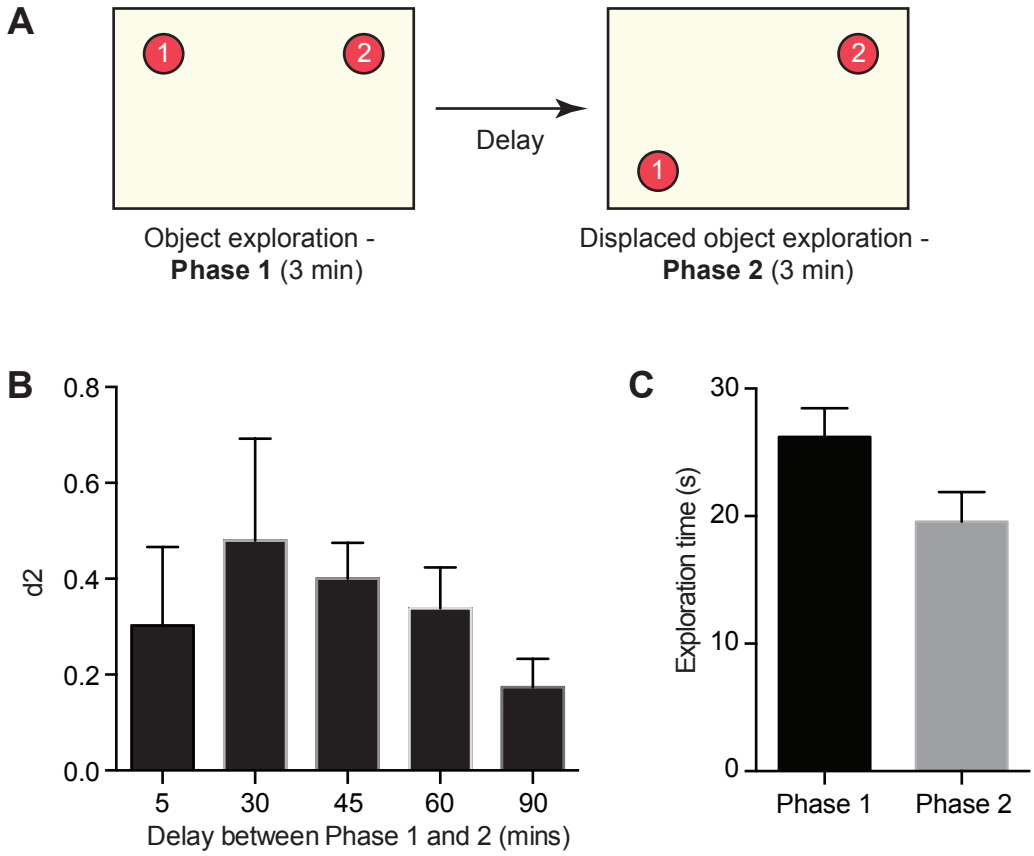


Figure 4.5: The spontaneous location recognition (SLR) task (A) Schematic of the SLR Task. During Phase 1, mice were placed in the middle of an open rectangular box with two identical objects placed in two corners of the box and were allowed to explore the objects for 3 minutes. Mice were removed and placed back in their home cage for the delay period. After the delay period was elapsed, the mice were placed back into the rectangular box with one of the objects displaced (Phase 2). The total time each mouse spent exploring each object was recorded. **(B)** The discrimination ratio (d2) for wild type mice with delay periods of 5 mins (n=4), 30 mins (n=5), 45 mins (n=8), 60 mins (n=6) and 90 mins (n=4). **(C)** Average value of the total time mice spent exploring both objects in Phase 1 and Phase 2. Error bars represent S.E.M.

4.3.1 Cholinergic activation during Phase 1 alter object preference in the SLR task

To determine the effects of cholinergic activation on different phases of SLR task, I implanted 17 WT and 13 ChAT-Ai32 mice with an optic fibre cannula in the MS. Previous reports have shown that administration of cholinergic agonists improves performance in various WM tasks (Deiana et al., 2011). Thus, I decided to use delay periods of 45 and 180 mins to determine if performance can be facilitated with cholinergic activation. To test these different stimulation protocols, 4 light stimulation conditions were examined: 'no light', light on for 'both phases', light on for 'Phase 1' only and light on for 'Phase 2' only. In total, each mouse performed 8 trials.

With 45 mins delay between Phase 1 and 2, control WT mice (n=17), on average, appear to spent more time exploring the displaced object during Phase 2 compared to the non-displaced object under all four light conditions, though only the 'no light' and 'Phase 1' light conditions were significant from chance exploration of d2=0 (one-sample *t* test: 'no light': $t(16)=3.054$, p=0.0076; 'Phase 1': $t(16)=2.387$, 0.0297; 'both phases': $t(16)=1.451$, p=0.17; 'Phase 2': $t(16)=1.27$, p=0.22). In contrast, ChAT-Ai32 mice (n=13) with cholinergic activation during 'Phase 1' on average spent a larger proportion of time exploring the non-displaced object (one-sample *t*-test compared to chance exploration of d2=0: $t(12)=2.363$, p=0.0358) while the other light condition groups explored the objects at chance (one-sample *t*-test compared to chance value of d2=0: 'no light': $t(12)=2.178$, p=0.05; 'both phases': $t(12)=0.625$, p=0.54; 'Phase 2': $t(12)=1.29$, p=0.22; Figure 4.6), suggesting that cholinergic activation during Phase 1 of the SLR task may alter object preference, and bias the preference for the non-displaced object. A similar trend was observed with 180 mins delay, however, none of the groups (WT or ChAT-Ai32) under any of the 4 light conditions explored the objects significantly from chance and thus, makes the results difficult to interpret (one-sample *t*-test compared to d2=0:

$p>0.05$ for all groups). Mixed design ANOVA with a within-subjects factor of light conditions ('no light', 'both phases', 'Phase 1' and 'Phase 2)' and delay (45, 180 mins) and a between-subjects factor of genotype (WT, ChAT-Ai32) revealed no main effect of genotype: $F(1,28)=3.389$, $p=0.0763$ (between-subjects effects), light: $F(3,84)=0.0975$, $p=0.4084$, nor delay: $F(1,28)=1.365$, $p=0.252$ (within-subjects effects). However, there was a significant effect of genotype and light interaction: $F(3,84)=2.721$, $p=0.0495$. There was no significant effect of genotype and delay interaction: $F(1,28)=1.719$, $p=0.2$, nor effect of light and delay interaction: $F(3,84)=1.083$, $p=0.361$. There was also no significant effect of genotype, light and delay interaction: $F(3,84)=0.362$, $p=0.781$.

To ensure that there was no object or place preference during Phase 1, I analysed the proportion of time the mice spent exploring object 1 during Phase 1 (p1). The data revealed no significant differences between the 8 groups (one-way ANOVA: $F(7, 258)=0.8288$, $p=0.5641$; Figure 4.6C) and p1 did not significantly differ from 0.5 (i.e. chance exploration; one-sample *t*-test compared to 0.5: $p>0.05$ for each of the 4 groups). Overall, this analysis shows that the mice show no object preference during Phase 1 and reassures that the increased preference for exploring the displaced subject during Phase 2 is most likely due to the displaced location.

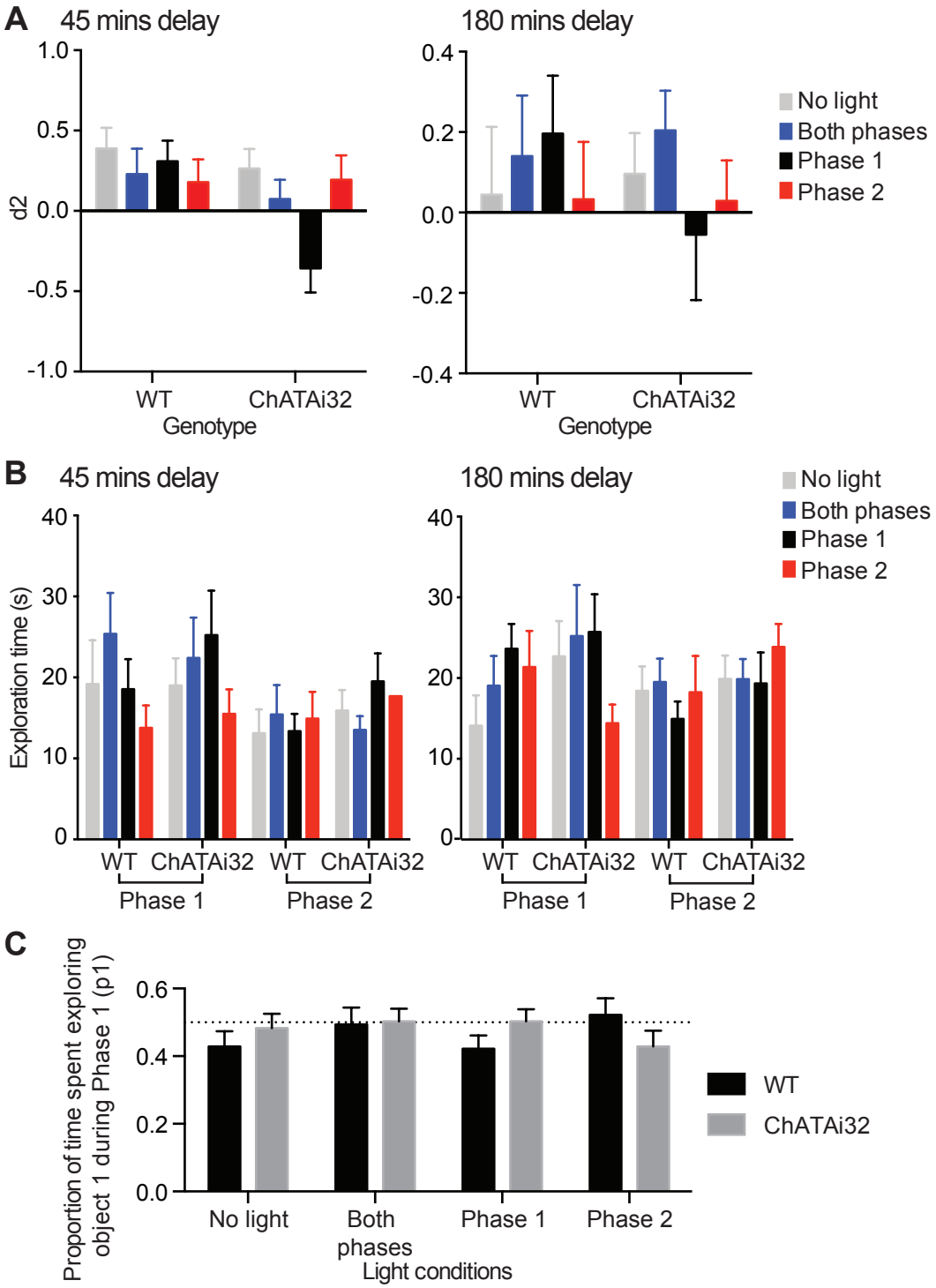
4.3.2 Cholinergic inactivation impairs performance in the SLR task

To determine the effects of cholinergic inactivation on different phases of this spatial WM task, I implanted ChAT-Ai40D mice with optic fibre cannula in the MS. Previous reports have demonstrated that ablation of MS cholinergic neurons impair spatial WM in this task (Okada et al., 2015). Therefore, I hypothesised that cholinergic inactivation would impair performance and as such, delay periods of 30 seconds, 5 minutes and 45 minutes were tested. Each

mouse also performed the task under the 4 light conditions ('no light', light on 'both phases', light on for 'Phase 1' only and light on for 'Phase 2' only).

With 30 seconds delay, the mice (n=5) under each of the 4 light conditions on average spent more time exploring the displaced object during Phase 2 compared to the non-displaced object. d2 significantly differ from 0 (i.e. mice exploring both objects equally) for light on during both phases (one-sample *t*-test: $t(4)=5.368$, $p=0.0058$), Phase 1 ($t(4)=5.181$, $p=0.0066$) and Phase 2 ($t(4)=21.66$, $p<0.0001$). In contrast, with 5 mins delay between Phase 1 and 2, the ChAT-Ai40D mice (n=24) only spent more time exploring the displaced object under the no light condition (one-sample *t*-test compared to d2=0: $t(23)=4.373$, $p=0.0002$; $p>0.05$ for the other light conditions).. Mice performed similarly with 45 mins delay (Figure 4.7). d2 significantly differ from 0 only for the no light condition (one-sample *t*-test: $t(23)=3.379$, $p=0.0026$; $p>0.05$ for the other light conditions). Repeated measures two-way ANOVA (within-subject factors of delay and light) revealed a main effect of light: $F(3,69)=4.863$, $p=0.00398$, but no main effect of delay: $F(1,23)=0.457$, $p=0.0506$ nor interaction between delay and light: $F(3,69)=0.069$, $p=0.976$. These findings suggest that cholinergic inactivation may impair performance on the SLR task, but given the large variability in the data, the results from these SLR experiments are difficult to interpret.

To ensure that there was no object nor place preference during Phase 1, I analysed the proportion of time the mice spent exploring object 1 during Phase 1 (p1). No significant differences were found between the 4 groups (one-way ANOVA: $F(3,198)=0.308$, $p=0.8196$) and it did not significantly differ from 0.5 (i.e. chance exploration; one-sample *t*-test compared to 0.5: $p>0.05$ for each of the 4 groups; Figure 4.7B). Overall, this analysis indicates that the mice show no object preference.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Phase 1
Phase 2
Light conditions
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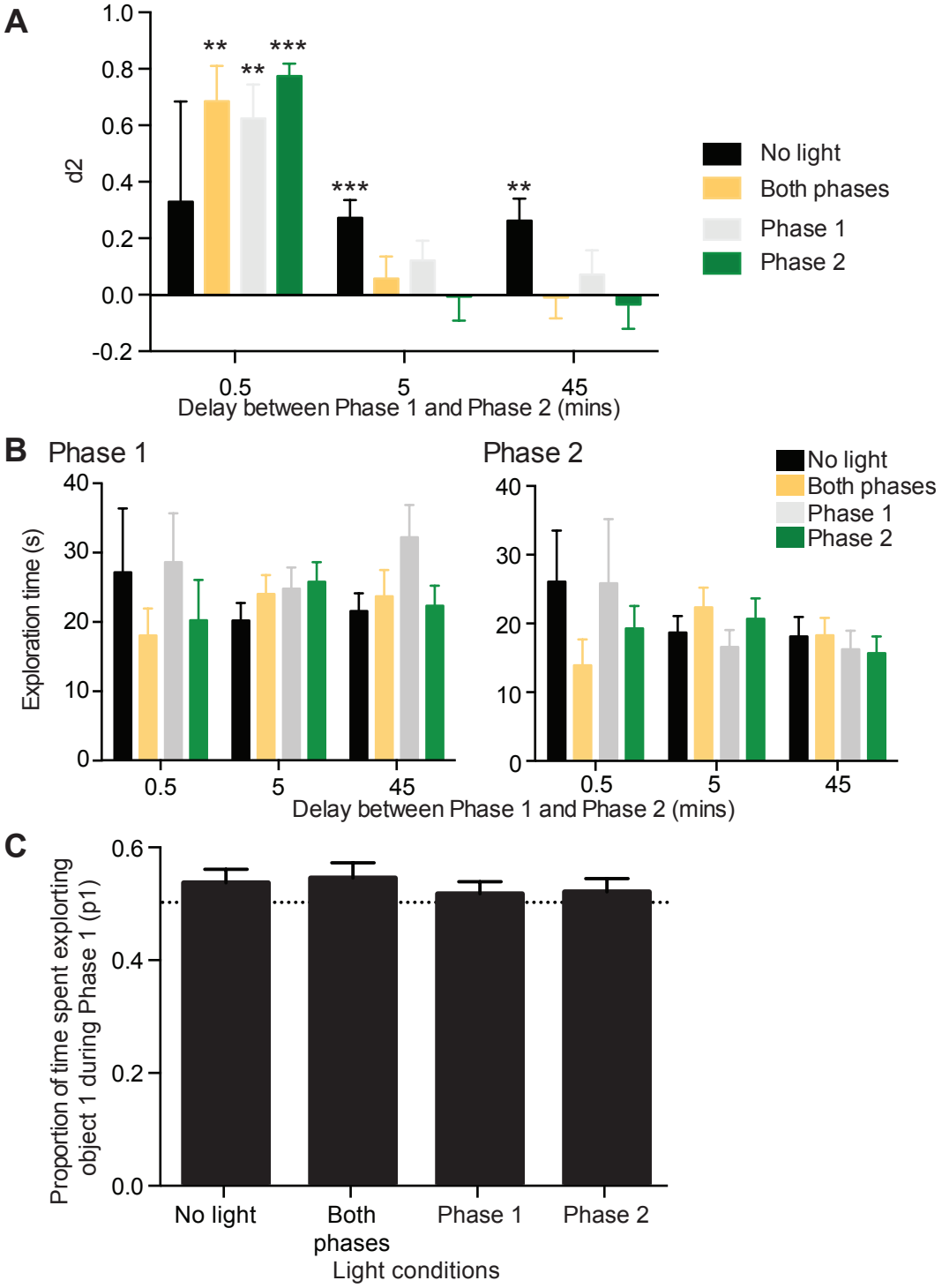
ChATAi32

Figure 4.6: Cholinergic activation during the Phase 1 appear to alter object preference in the SLR task **(A)** The discrimination ratio (d2) for wild-type (WT; n=17) and ChAT-Ai32 mice (n=13) with a delay period of 45 mins (left) and 180 mins (right) under 4 light conditions: no light (grey); light on during both phases (blue); light on during Phase 1 only (black); light on during Phase 2 only (red). **(B)** Average value of the total time mice spent exploring both objects in Phase 1 and Phase 2 for each group mice under 4 light conditions with a delay period of 45 mins (left) and 180 mins (right). **(C)** The proportion of time (p1) WT and ChAT-Ai32 mice spent exploring object 1 during the Phase 1 under the 8 groups. There was no significant difference between the two genotypes of mice. Broken line represents chance exploration of 0.5. Error bars represent S.E.M.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Phase 2
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Proportion of time spent exploring object 1 during Phase 1 (p1)
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No light
Both phases
Phase 1
Phase 2
Light conditions

Figure 4.7: Cholinergic inactivation impaired performance in the hippocampus-dependent SLR task. (A) The discrimination ratio (d2) for ChAT-Ai40 mice with delay periods of 30 s (n=5), 5 mins (n=24) and 45 mins (n=24) under 4 light conditions: no light (black); light on during both phases (yellow); light on during the Phase 1 only (grey); light on during Phase 2 only (green). **p<0.01 ***p<0.001; one-sample *t*-test compared to chance exploration (d2=0). **(B)** Average value of the total time mice spent exploring both objects in Phase 1 (left) and Phase 2 (right) for each group mice under 4 light conditions with a delay period of 30 s, 5 mins and 45 mins. **(C)** The proportion of time mice spent exploring object 1 during Phase 1 under the 4 different light conditions. Broken line indicates chance exploration of 0.5. Error bars represent S.E.M.

4.4 Discussion

In the present study, I used optogenetics to activate or inactivate the cholinergic system and tested its effects on WM in the spontaneous alternation T-maze and the SLR task. During the spontaneous alternation T-maze task, the alternation rate was lower than the expected alternation rate in published studies (Deacon and Rawlins, 2006) and, as such, the results are difficult to interpret. Cholinergic inactivation appears not to have an effect on spontaneous alternation, but the sample size may be too small to observe an effect. In the SLR task, the cholinergic system appears to have an ambiguous effect on WM. Cholinergic activation during Phase 1 appears alter object preference while cholinergic inactivation may impair performance, suggesting that suboptimal levels of ACh (either too much or not enough) could affect WM.

4.4.1 Technical considerations

When evaluating the results presented in this chapter, it is important to consider whether or not the experimental approach used is appropriate for addressing the research questions and that the results can be reproduced reliably.

4.4.1.1 Choice of behavioural tasks

The spontaneous alternation T-maze task

This task was first chosen to test the effects of cholinergic modulation on WM. It is a commonly used task and is considered to be one of the most sensitive spatial memory tasks used to detect hippocampal dysfunction (Deacon and Rawlins, 2006). It also has the advantage of being a dry land task which eliminates the stress of forced swimming displayed by mice (Whishaw and Tomie, 1996).

Spontaneous alternation reflects the motivation of mice to explore their environment and mice show alternation behaviour without any food or water deprivation. While the T-maze has been used for decades in academia and industry, it is also known that it can be unreliable; typical alternation rates are around 75-80% with no time delay between Phase 1 and 2 (Dember and Fowler, 1958; Deacon and Rawlins, 2006). However, in the current study with ChAT-Ai32 mice (Figure 4.2), the alternation rate was much lower. Another limitation of this task is that the performance of mice is not only affected by WM function, but also by novelty seeking and the exploratory behaviour of the mice. As this task is based on spontaneous exploratory activity, it is possible that individual animals develop a preference for a specific place that is independent of the familiarity/novelty of the environment. Indeed, most of the mice had an arm preference, choosing the same arm in over 50% of trials during Phase 1. The T-maze was placed in the centre of the testing room and there was no visible discernible difference between either arms of the T-maze. The experimental equipment set-up was the same as that in the pilot experiment (Figure 4.1) during which the alternation rates was comparable to the expected rate. Further analysis of the mice's performance suggests that alternation rate decreases as the trial period elapses; as the environment became familiar over the period of 120 trials, their motivation for exploration faded (Figure 4.3).

Given the large amount of data needed to be collected for each mouse, rewarded (instead of spontaneous) alternation would perhaps have been the more appropriate choice as mice could run up to 20 trials per day or more if not sated (Deacon and Rawlins, 2006). However, as the mice were used to perform a number of different tasks presented in this thesis, spontaneous alternation was preferred. As spontanoues alternation is not a 'learned' behaviour and was not reinforced with reward, these mice could be used for further testing in other tasks without interference. Another solution to overcome this low spontaneous alternation issue could be to use a larger sample size and split the cohort into 4 groups to test each light condition or split the cohort into 3 groups to test each

delay condition separately. As such, for the study with ChAT-Ai40D mice, I split the cohort into 4 groups and each mouse performed fewer trials (20). Indeed, the spontaneous alternation rate was higher and was similar to the expected 75-80% with no delay (Figure 4.4).

The spontaneous location recognition task

Mice were also tested on the SLR task to further investigate the effects of cholinergic modulation on WM. Like the alternation T-maze task, the SLR task is another hippocampus-dependent spatial memory task that harnesses the rodents' natural strong novelty preference and is known to depend on cholinergic neurons in the MS/vertical diagonal band (MS/vDB; Okada et al., 2015). To maintain interest, each mouse was presented with a pair of new objects for each trial.

The amount of time spent exploring each object was determined manually, recorded using JWatcher V1.0, written in Java^{TM}. In an effort to be consistent and unbiased, a rigorous criteria was defined for exploration – the mouse must direct its nose to the object at a distance of 2 cm or less; climbing, sitting, or chewing on the object was not included as exploratory behaviour (as also defined in other published studies such as Kent et al., 2015). To minimise bias, each trial was recorded and analysed at least 7 days after the trial. The recordings were labelled blind (i.e. I did not know which mouse was performing the task under which light condition) and analysed in a pseudorandom order. Nevertheless, there may be variation in the analysis which can greatly vary the results. A better method for analysis might have been to use a tracking software that tracks the movement of the mice in the test arena and can automatically determine the amount of time spent exploring each object by each mouse and, as such, remove any bias that may be introduced by the experimenter.

Nevertheless, as this task is based on spontaneous exploratory activity, individual animals could have a preference for a specific place that is independent of

the familiarity/novelty of object location. To ensure that there was no object preference, the proportion of time the mouse spent with one of the two objects presented during Phase 1 was analysed and mice on average spent an equivalent period of time exploring both objects during Phase 1. There was also no significant difference from 0.5 (i.e. chance exploration).

4.4.1.2 Statistical tests

Another advantage of spontaneous tasks is that each mouse could be tested under each light condition and thereby statistical power was increased. For the spontaneous alternation T-maze task performed in ChAT-Ai32 mice, when comparison was within subjects, repeated-measures one-way ANOVA was used. With ChAT-Ai40D mice, since comparison was between subjects, as mice were evenly split into 4 groups to test each of the light conditions, one-way ANOVA was performed.

For the SLR task performed with WT and ChAT-Ai32 mice, there were two factors to be considered - genotype of mice and light conditions. While the independent variable 'genotype' was between subjects, the independent variable 'light conditions' was within subjects and as such, a mixed design ANOVA was used to test whether light and/or genotype had an effect on SLR task performance. As there was a significant effect of genotype, to further explore this effect, a repeated-measures one-way ANOVA was then used to test whether the difference in the discrimination ratio (d2) under the light conditions within each genotype was significant. For ChAT-Ai40D mice, the repeated measures one-way ANOVA was again used as there was only one factor to be considered - light conditions, within subjects.

4.4.2 A role for MS cholinergic neurons in working memory

The results in this chapter are inconclusive. The effects of cholinergic modulation on WM may be subtle, further exploration is required. Some previous studies have implicated MS/vDB cholinergic cells in spatial WM but the data are also mixed and inconclusive. The results may vary due to experimental design, species and choice of behavioural tests.

Pharmacological manipulations of cholinergic transmission have revealed heterogenous effects on spatial learning. In humans, scopolamine (mAChR antagonist) failed to cause deficits on WM (Beatty, 1986). While systemic intraperitoneal and subcutaneous dosing of nicotine (nAChR agonist) enhanced WM performance, oral or locally administered nicotine (such as into the ventral hippocampus) did not lead to any effects on WM performance in rodents (Kim and Levin, 1996). On the other hand, block of cholinergic transmission via either nAChR or mAChR antagonists in the ventral hippocampus caused significant deficits in the accuracy of WM performance on the radial-arm maze (Kim and Levin, 1996). This differential effect suggests a fundamental role for nAChRs and mAChRs in spatial WM but a high dose of agonists may be required to induce comparable memory enhancement. That being so, the administration of broadly active agonists may not be of ubiquitous therapeutic relevance and requires refinement. WM deficits dependent on age have been shown to ameliorated by nicotine but again, this effect is dose and task-dependent (Bontempi et al., 2001).

Moreover, during spontaneous alternation/WM tasks, levels of hippocampal ACh increase (Fadda et al., 2000). Increased ACh release has also been reported in the cortex during alternations tasks (Giovannini et al., 1998; Anzalone et al., 2009). *In vivo* microdialysis studies looking into the relationships between prefrontal and hippocampal ACh systems and WM by varying task demands revealed that cortical ACh was released during WM only, hippocampal ACh was

mostly related to long-term reference memory (Hironaka et al., 2001).

Furthermore, immunotoxic lesions (via cholinergic ablation with 192-IgG saporin) in the MS/vDB have revealed spatial WM deficits in the SLR task (Perry et al., 2001; Okada et al., 2015); ablation of striatal cholinergic interneurons also impairs performance in the alternation T-maze task. However, other lesion studies have reported no significant effects of immunotoxic lesions on spatial memory performance (Torres et al., 1994; Baxter and Gallagher, 1996; Baxter et al., 1996; McMahan et al., 1997; Vuckovich et al., 2004). This discrepancy could be due to differences in the administration, dose and spread of the immunotoxins. Widespread intraventricular administration of 192 IgG-saporin have been shown to not only damage basal forebrain cholinergic neurons, but also cerebellar Purkinje cells that resulted in severe motor deficits (Heckers et al., 1994; Waite and Thal, 1995; Waite et al., 1999). As such, spatial learning deficits may not be purely due to loss of MS/vDB cholinergic neurons. However, more selective lesions may not be sufficient to induce impairments as Moser et al. (1993) found that in order to induce spatial learning impairments, at least 20% of the hippocampal volume must be damaged. In addition, it has been suggested that multiple strategies can be employed to solve spatial problems (Whishaw et al., 1995; Dudchenko et al., 1997; Martin et al., 1997), only some of which are affected by ablation of the hippocampal cholinergic input. Some studies have indicated that lesions of septal cholinergic neurons bias rats away from using an allocentric strategy to learn the radial arm maze task (Janis et al., 1998; Lehmann et al., 2003). Further examination of particular strategies used by rodents to solve different spatial tasks may be a worthwhile avenue of exploration as selective inactivation of various inputs into the hippocampus may reveal how spatial cognition may be fractionated within the hippocampus.

In summary, all these studies reveal that the effects of the cholinergic system on WM is complex. The precise role of ACh in WM is not well-understood partly due to the large variability in the response to cholinergic modulators both across

different behavioural tasks and across different experimental setups. It has been suggested that there is an inverted U-shape relationships between cognitive performance and neuromodulatory activity (Yerkes and Dodson, 1908; Clatworthy et al., 2009; Cools and D'Esposito, 2011), where levels of neuromodulators that are either too low or too high both impair performance. The mixed data in the literature and findings in this chapter where cholinergic activation and inactivation both impaired performance in the SLR task (Figures 4.6 and 4.7) appear to support this hypothesis. Further investigation of this relationship with microdialysis experiment and a dose-response design with various ACh agonists and antagonists will be necessary to confirm this hypothesis.

4.4.3 Perspective

This chapter provides some new insights into the role of the cholinergic system in WM. These findings also highlight the importance of being mindful of logistics when designing and choosing behavioural tasks best suited to test an hypothesis. While it is well known that rodents have strong preferences for novelty, performing 120 trials in the same environment means that the environment is no longer novel and mice no longer spontaneously alternate. Thus, the research may not be reproducible. To more reliably test the effects of cholinergic system in WM, a rewarded task may be more appropriate.

The differential effects of cholinergic manipulations on tasks with distinct cognitive demands (Shen et al., 1996; Nicholls et al., 2008; Hironaka et al., 2001) indicate that the cholinergic system affects short- and long-term hippocampus-dependent memory by distinct neural mechanisms. Therefore, in the next chapter, I investigated whether there were differential effects of cholinergic modulation in different phases of hippocampus-dependent long-term memory tasks.

Chapter 5

Neuromodulation of long-term memory processes

5.1 Introduction

Long-term memory formation is thought to rely on a two-stage process where new information is first encoded and then consolidated (Dudchenko, 2004; Cowan, 2008; Aben et al., 2012). Memory encoding is associated with theta and gamma oscillations where they are thought to help coordinate activity between different brain regions for sensory information processing and facilitate accurate spike timing for synaptic plasticity. Memory consolidation is dominated by SWRs, throughout which reactivation of previously encoded sequences occurs.

Neuromodulators, particularly ACh, play a central role in shifting states of the brain. High levels of ACh are observed during exploration while ACh levels are low during subsequent rest (Fadel, 2011). As observed in chapter 3 and by Vandecasteele et al. (2014), activation of septal cholinergic input shifts brain states from SWRs to theta oscillations. The behavioural implications of this ACh-facilitated SWR suppression remain to be investigated.

At the level of cellular networks, changes in synaptic efficacy (LTP and LTD) play a central role in learning and memory (Hughes, 1958; Colicos and Syed, 2006; Zenke et al., 2015). ACh and DA exert major modulatory inputs in synaptic plasticity in the hippocampus. ACh has been shown to favour synaptic depression (Brzosko et al., 2017). Recent data showed that t-LTP can be converted to t-LTD with the application of ACh before and during the t-LTP pairing induction protocol (Brzosko et al., 2017). Furthermore, it was demonstrated that ACh output in the dorsomedial striatum selectively increased during the acquisition of a reversal learning task, but not during the initial place learning acquisition (Ragozzino et al., 2009). How critical the role of ACh in LTD induction and consequent behaviour still needs to be elucidated.

DA, on the other hand, favours the induction of LTP. DA can extend the time window for t-LTP induction (Zhang et al., 2009; Ruan et al., 2014; Yang and Dani, 2014) and rescue sucrose-induced t-LTP deficit (Edelmann and Lessmann, 2011), and application of DA before, during and immediately after the t-LTD pairing protocol can retroactively convert t-LTD to t-LTP (Brzosko et al., 2015). Furthermore, DA or DA agonists enhance memory and selective depletion, dysfunction, or lesion of forebrain DA systems bring about memory acquisition and retention deficits. Hyperdopaminergia in dopamine transporter knock-out mice severely impairs hippocampal LTD that is associated with impaired reversal learning (Morice et al., 2007). These impairments can be reversed by the dopamine receptor antagonist haloperidol. Moreover, phasic firing of VTA DA neurons alone has been demonstrated to be sufficient for behavioural conditioning (Tsai et al., 2009) and augments the choice preferences for the DA stimulation (Stauffer et al., 2016). Thus DA has been proposed to be a powerful reward reinforcement signal. However, whether transient DA can also drive and enhance goal-directed learning and its effects on spatial reversal learning in a hippocampus-dependent spatial memory task remain to be determined.

5.1.1 Aim

I sought to find a behavioural correlate to the observed effects of ACh and DA on hippocampal LFP in chapter 3 and on alterations of hippocampal synaptic plasticity *in vitro* by Brzosko et al. (2015, 2017). I hypothesised that:

1. cholinergic activation during consummatory behaviour could impair performance in a long-term spatial memory task; as during pauses in locomotion such as consummatory behaviour, awake SWRs that have been suggested to contribute to memory consolidation (Jadhav et al., 2012) could be suppressed by cholinergic activation (Vandecasteele et al., 2014)

2. the absence of ACh by cholinergic inactivation would impede reversal learning. Since ACh favours synaptic depression (Brzosko et al., 2017), inactivation of ACh neurons may impair synaptic depression, which promotes the extinction or reversal of memories formed earlier (Nicholls et al., 2008)

3. dopaminergic activation could enhance or even drive goal-directed place learning because DA has been shown to facilitate induction of LTP (Brzosko et al., 2015), and

4. dopaminergic activation could disrupt reversal learning because DA has been shown to enhance LTP (Yao et al., 2004) and can convert t-LTD to t-LTP (Brzosko et al., 2015) and, as such, dopaminergic activation may impair LTD required for reversal learning (Morice et al., 2007).

To test the first hypothesis, I implanted ChAT-Ai32 mice with optic fibre cannulae in the MS to activate septal cholinergic neurons. These mice were tested on the widely-used appetitive Y-maze task that was divided into two phases to probe the differential effects, if any, of cholinergic activation during exploration (memory encoding) and reward (memory consolidation).

The appetitive Y-maze task is a simple spatial memory task with a single choice point with two alternatives. A slightly more complex task that offered two different starting points and two options for reward locations was required to test reversal learning. A common task to test spatial reversal learning in rodents is the Morris water maze task (Duffy et al., 2008; Morice et al., 2007), but its adaptation for mice is associated with problems. In particular, swimming in water is an aversive activity for mice and the impact of stress must be taken into account in the assessment of cognitive performance. Forced swimming was demonstrated to alter various neurochemicals (Kirby et al., 1997), which could interfere with examining cognitive function (Patil et al., 2009). To avoid the stress induced by swimming, I developed an appetitively-motivated dry-land

task that consists of a circular open field (OF).

This task was first validated with WT mice. Subsequently, to test the remaining hypotheses, I implanted ChAT-Ai40D and DAT-Ai32 mice with optic fibre cannulae in the MS to inactivate septal cholinergic neurons, or the VTA to activate VTA dopaminergic neurons, respectively. These mice were food-restricted, habituated to the food reward (sweetened condensed milk) and OF. Their task performance was then evaluated during place and reversal learning. To test whether dopaminergic activation alone was sufficient for goal-directed learning, an additional group of DAT-Ai32 mice were added during the place learning phase of the task where the mice did not receive any food reward, but received dopaminergic activation at the reward location instead.

5.2 Spatial learning on the appetitive Y-maze task

The appetitively-motivated hippocampus-dependent Y-maze task is a commonly used task to study long-term spatial memory (von Engelhardt et al., 2008; Brown-borg et al., 2010; Bannerman et al., 2012; Shipton et al., 2014). I first chose this established task to investigate the effects of cholinergic activation on long-term memory performance. Mice were food-deprived and habituated to the elevated Y-maze. In this task, mice had to learn to find a food reward on an elevated 3 armed Y-maze which remained at a fixed location in relation to visual cues in the room. Short-term memory errors caused by arm re-entry during a single trial have previously been shown to impair the acquisition of spatial long term memory task (Schmitt et al., 2003). Thus, to isolate the long-term memory process in this task, mice were only allowed to make one arm choice each trial; mice were allowed to consume the reward if they chose the correct arm but if they chose the wrong arm, mice were taken out of the maze and the trial was ended (i.e. mice were not permitted to self-correct, Figure 5.1A;

Shipton et al., 2014).

Mice received 10 trials daily and, between each trial, the maze was rotated 120^o clockwise or anti-clockwise so that mice could not use intra-maze cues to perform the task successfully (Deacon et al., 2002; Reisel et al., 2002). Mice started five trials from the arm to the left of the designated rewarded arm, and five from the right, in a pseudorandom sequence (Figure 5.1B). This was repeated for at least 10 consecutive days and the number of correct arm choices was recorded. On the final day of testing, to ensure that mice did not use olfactory cues to navigate to the reward, food was delivered only once the mice had made their arm choice (post-choice baiting, P.C.B.).

Given a previous study reporting abnormal behaviour in ChAT-Cre mice (Kolisnyk et al., 2013), I wanted to first verify that the performance of male heterozygous ChAT-Cre mice (Jackson Laboratories, stock #006410, n=8) was similar to that of control mice (negative littermates or WT C57BL/6J mice bought from Jackson Laboratories, n=7) on the appetitive Y-maze task (Figure 5.1C and D). Mice in both groups acquired the task at a similar rate over the course of testing, reaching 90–100% accuracy by day 7, suggesting that this line of ChAT-Cre mice has normal long-term spatial memory.

5.2.1 Cholinergic activation during reward impedes place learning

One of the main treatments for cognitive dysfunction is to pharmacologically increase levels of ACh (Colović et al., 2013), this increase is typically tonic and timing independent. However, given the dichotomy of ACh effects on memory formation, where high concentration of ACh during exploration promotes theta and gamma oscillations and thereby facilitates memory encoding while low levels of ACh during subsequent rest enables SWRs that promote memory con-

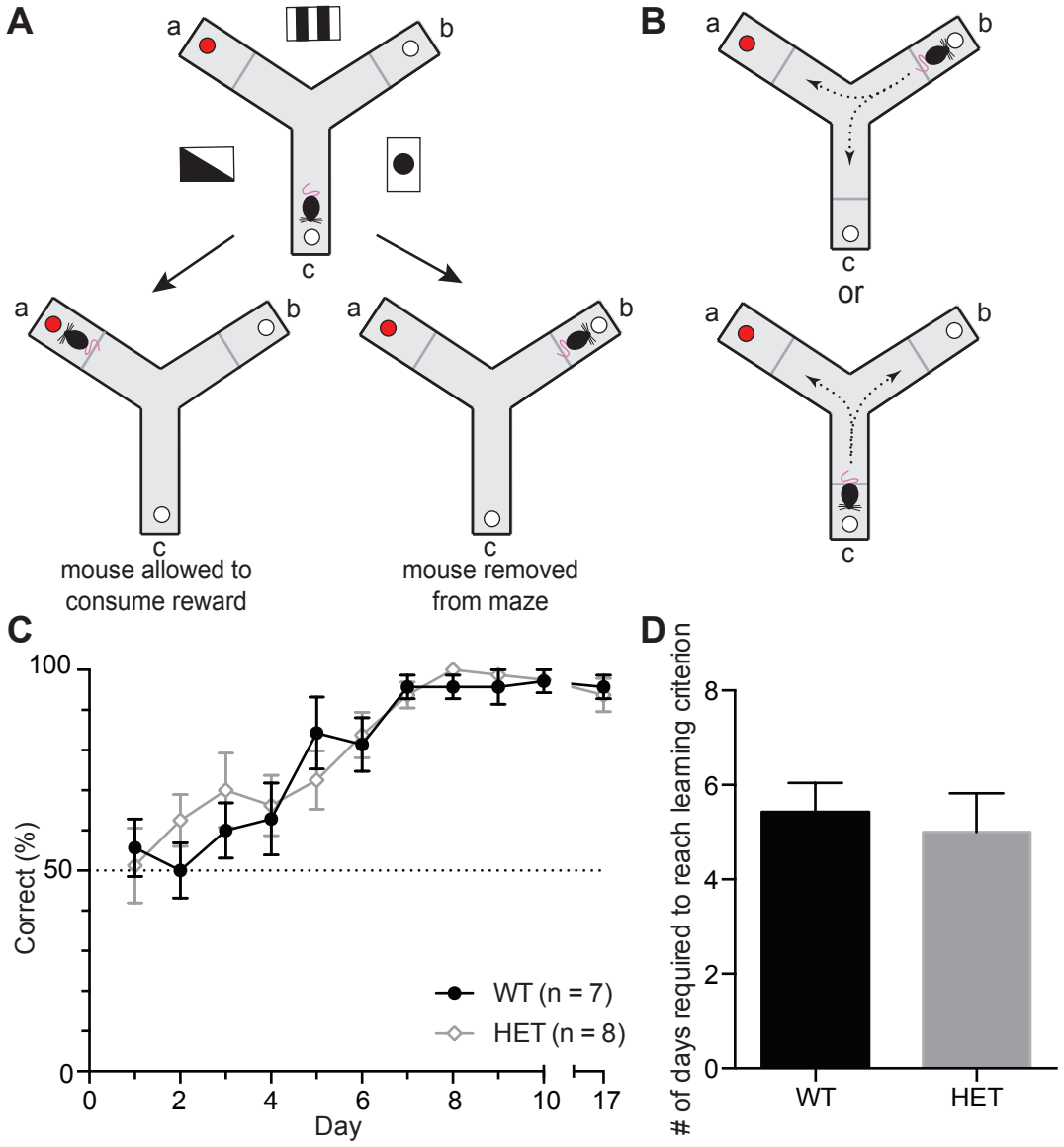

Figure 5.1: The Appetitive Y-maze task (A) Mice were trained to find the food reward (red dot) on an elevated 3 arm Y-maze which remained at a fixed location in relation to visual cues in the room. Mice were allowed to consume the reward if they chose the correct arm but if they chose the incorrect arm, mice were removed from the maze. **(B)** Mice started in the other two arms in a pseudorandom sequence. **(C)** Mice received blocks of 10 trials a day for 10 consecutive days. They were tested a week later to ensure retention of long-term memory. The number of correct arm choices was recorded. The performance of both WT (n=7) and heterozygous ChAT-Cre mice (n=8) improved over the testing period, to a similar extent. Dashed line indicates chance performance of 50%. **(D)** The average number of days required for each group to reach learning criterion (>80%). Error bars represent S.E.M.

solidation (Hasselmo and Mcgaughy, 2004), a tonic increase in ACh levels may not always be beneficial and may have contradictory effects on learning and memory. Therefore, I sought to investigate the effects of cholinergic activation on various phases of the appetitive Y-maze task, dividing the task into two stages: exploration and reward. Grey tape marked 20 cm from the distal end of the arm indicating the division of the two processes. Cholinergic activation was achieved by light stimulation of ChR2. Light (25 mW, 473 nm) was delivered as 50 ms-long pulses at 10 Hz frequency.

Thirty-six ChAT-Ai32 mice (offspring of ChAT-Cre mice crossed with the Cre-reporter Ai32 line bearing eYFP-tagged ChR2) implanted with an optic fibre in the MS were evenly split into four groups (i.e. n=9 in each group) to test four light stimulation conditions: (i) 'no light', (ii) light stimulation 'throughout the maze', (iii) light stimulation during 'exploration' only - from start of trial until the grey tape of their chosen arm and (iv) light stimulation during 'reward' only - from the grey tape until the mice were either removed from the maze if they chose the incorrect arm or until the mice finished consuming the food reward if they made the correct choice. The performance of all four groups of mice improved but at different rates (Figure 5.2A). The number of days required to reach learning criterion of at least 80% accuracy for the remainder of the testing period was determined ('no light' = 3.33±0.33 days, 'throughout maze' = 3.56±0.41 days, 'exploration' = 3.11±1.05 days, 'reward' = 5.22±1.56 days to reach learning criterion) and one-way ANOVA revealed significant differences between the groups ($F(3,32)=5.473$, $p=0.004$; Figure 5.2B). As sample sizes were equal, Tukey post-hoc test was used and it revealed significant differences between the 'reward' group and: the 'no light' group ($p=0.0097$); 'throughout maze' group ($p=0.0386$); 'exploration' group ($p=0.006$). This suggests that cholinergic activation during reward slows learning of the appetitive Y-maze task while light stimulation during exploration or continuous activation has no significant effect on task acquisition. The difference in performance observed between the 'reward' and 'throughout maze' cohorts could be due to fatigue of

the light response with continuous stimulation. While cholinergic activation shifts brain states from SWR state to theta-gamma state that persists for the duration of the light stimulation (Figure 3.4), it is unclear for how long after the start of light stimulation, activated cholinergic neurons continue to release ACh and, as such, the effects of cholinergic activation in the 'throughout maze' cohort may not be as prominent as those observed in the 'reward' group. *In vivo* LFP recordings during the task, along with voltammetry, could be performed to verify this hypothesis.

Furthermore, while the light stimulation during reward impairs the learning curve, it has no significant effect on the retention. When the mice were tested again 7 days later, task performance between the 4 groups was not significantly different ('no light' = 100%, 'throughout maze' = 100%, 'exploration' = 96.33±1.11%, 'reward' = 84.44±3.38%; one-way ANOVA: $F(3,32)=2.211$, $p=0.106$).

To ensure that the deficit was not simply due to effects of light during reward, WT mice (negative ChAT-Ai32 or C57BL/6J mice) were also tested using the same lighting paradigm. All WT mice learnt the task (Figure 5.2C, D) and when compared to the learning performance of ChAT-Ai32 mice, mixed-design ANOVA with a within-subjects factor of day (1–10) and between-subjects factors of genotype (WT, ChAT-Ai32) and light ('no light', 'throughout maze', 'exploration', 'reward') revealed main effects of day: $F(9,400)=16.52$, $p<0.001$ and a significant interaction between genotype and light: $F(3,400)=11.74$, $p<0.001$. There was no significant difference in genotype: $F(1,400)=0.69$, $p=0.407$, nor light: $F(3,400)=0.986$, p-0.399.. Although the sample size of WT mice was small, there was no indication of impaired performance in the light on during reward cohort.

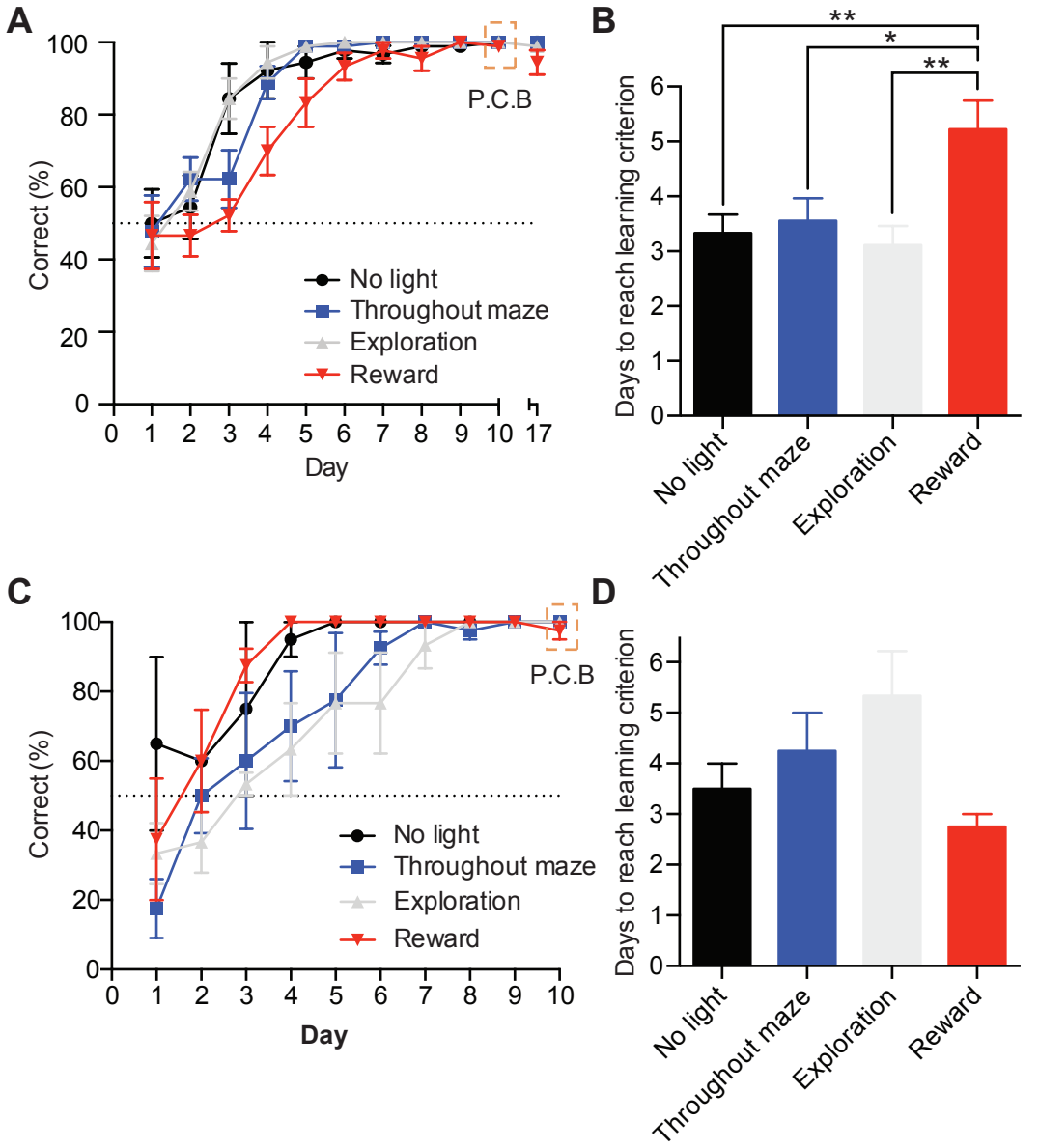

Figure 5.2: Cholinergic activation during reward slows learning on the appetitive Y-maze task. (A) ChAT-Ai32 mice were split into 4 groups to test 4 light conditions: no light (black, n=9); light on throughout maze (blue, n=9); light on during exploration only (grey, n=9); and light on during reward only (red, n=9). They received blocks of 10 trials a day for ten consecutive days and the number of correct arm entries were recorded. On the tenth day, the reward was given after arm choice (box labelled P.C.B.). Testing was paused for 7 days to test memory retention. The performance of all four groups of mice improved but at a different rate. **(B)** The average number of days required for each group of ChAT-Ai32 mice to reach learning criterion of at least 80%. *$p<0.05$; **$p<0.01$. **(C)** Wild-type mice were also split in 4 groups to ensure that the effects observed in ChAT-Ai32 mice were due to stimulation of ChR2 and not simply effects of light (no light, n=2; throughout maze, n=4; exploration, n=3; reward, n=4). Dashed lines represent chance performance of 50%. **(D)** The average number of days required for each group of wild-type mice to reach learning criterion. Error bars represent S.E.M.

5.3 Spatial learning in an open field

While the Y-maze is a well-known spatial long-term memory task that is known to depend on the hippocampus, it is a simple task with a single choice point and two alternative choices. To mimic a more natural navigation environment and to add an option to test reversal learning, I created an appetitively-motivated long-term spatial memory task based in a circular OF.

Similar to the appetitive Y-maze task, mice were food-deprived and first habituated to the OF. Mice were required to learn the location of the reward that remained constant in relation to visual cues in the room. However, rather than following a limited path, the mice were free to explore the entire OF. The OF was divided into four quadrants, each of which were further divided into an inner quadrant and an outer quadrant. The mice were still presented with a binary choice of two food wells in opposite inner quadrants (Figure 5.3Ai). Mice were only allowed to make one food well choice in each trial; if the mouse entered the correct inner quadrant (i.e. the quadrant containing a food well with food reward), it was allowed to consume the food reward, but if the mouse entered the incorrect inner quadrant containing an empty food well, the mouse was removed from the OF and the trial ended.

Each mouse received blocks of 10 trials daily and between each trial, the maze was rotated 90^{o} clockwise or anti-clockwise so that mice could not use intra-maze cues to perform the task successfully (Deacon et al., 2002; Reisel et al., 2002). Mice, facing outwards, started five trials from the outer quadrant to the left of the designated rewarded quadrant, and five from the right in a psedo-random sequence (Figure 5.3Aii). This was repeated for at least 8 days and the number times the mice reached the correct reward location was recorded. The search paths the mice took were analysed and the time spent at each quadrant or the distance travelled on the OF during each trial was determined.

To validate this behavioural testing paradigm and build a baseline for learning performance, I first tested the task using WT mice (n=6). Their performance improved over 8 days (Figure 5.3), requiring an average of 5.33±1.17 days to reach learning criterion of 80% for the remainder of the testing period.

This OF paradigm also allowed the opportunity to probe reversal learning. During the reversal learning phase, the reward was moved to the opposite quadrant, reversing the reward values of the food wells. The mice started in the same two quadrants as during the learning phase in a pseudorandom order (Figure 5.4A).

Of the mice that learnt the new location (n=4), they reached learning criterion in an average of 6.75±0.85 days (Figure 5.4). Two mice did not learn the new location after 12 days. Interestingly, mice that learnt the initial location most quickly during the place learning phase appear to take the longest to learn the new location during reversal learning (Figure 5.4E).

5.3.1 Cholinergic inactivation appears to impair reversal learning

LTD has been suggested to be the cellular mechanism that underlies the behavioural flexibility required for spatial reversal learning. Enhanced LTD is linked with an improvement in spatial reversal learning. Application of the NMDA receptor co-agonist, D-serine enhances LTD without affecting potentiation in acute hippocampal slices (Duffy et al., 2008). Correspondingly, systemic administration of the same co-agonist enhances performance during the reversal phase of the Morris water maze task, but does not affect the initial acquisition (Nicholls et al., 2008). Recent data showed that application of ACh facilitated t-LTD (Brzosko et al., 2017) and thus I hypothesised that cholinergic inacti-

vation would impair and slow reversal learning but not the initial place learning.

To test this hypothesis, I tested ChAT-Ai40D mice on the OF to probe the effects of cholinergic inactivation on long-term place and reversal learning. Cholinergic inactivation was achieved by light stimulation of ArchT. Light was delivered for the duration of each trial (10 seconds to 2 minutes) by manual control of the shutter. ChAT-Ai40D mice were divided into two groups: 'no light' stimulation as the control group (n=4) and continuous 'light on' stimulation throughout the trial (n=5). I first trained the mice to find the food reward located in a fixed location of the OF. All of the mice in both the 'no light' and 'light on' groups learnt the task and at an equivalent rate (Figure 5.5B, days 1–8), reaching the learning criterion of at least 80% for the remainder of the place learning phase in an average of 5.25±1.1 and 4.6±0.81 days respectively (one-tailed Mann-Whitney test: p=0.35, Figure 5.5C). As the mice learnt the task, the distance they travelled on the OF decreased (Figure 5.5A and D). Analysis of the difference in path lengths between the first and last day of the place learning phase revealed no significant difference between the two groups ('no light' = –140.5±71 cm; 'light on' = –67.9±26 cm; one-tailed Mann-Whitney test: p=0.28, Figure 5.5D).

The reward was then moved to the opposite quadrant and the mice were trained to go to this new location (Figure 5.5B, days 9–20). While every mouse in the 'no light' group learnt the new location, one of the five mice in the 'light on' group did not. Furthermore, the 'light on' group (the group with cholinergic inactivation during the task) acquired this new location more slowly, requiring a mean of 9.6±1.3 days to reach learning criterion (at least 80% accuracy for the remainder of the reversal learning phase, mice that did not learn was listed as 13 days) compared to 6±0.4 days for the control 'no light' group (one-tailed Mann-Whitney test: p=0.03).

Just as observed during place learning, as the mice learnt the reward location, their path lengths decreased; mice took the shortest path to reward from start

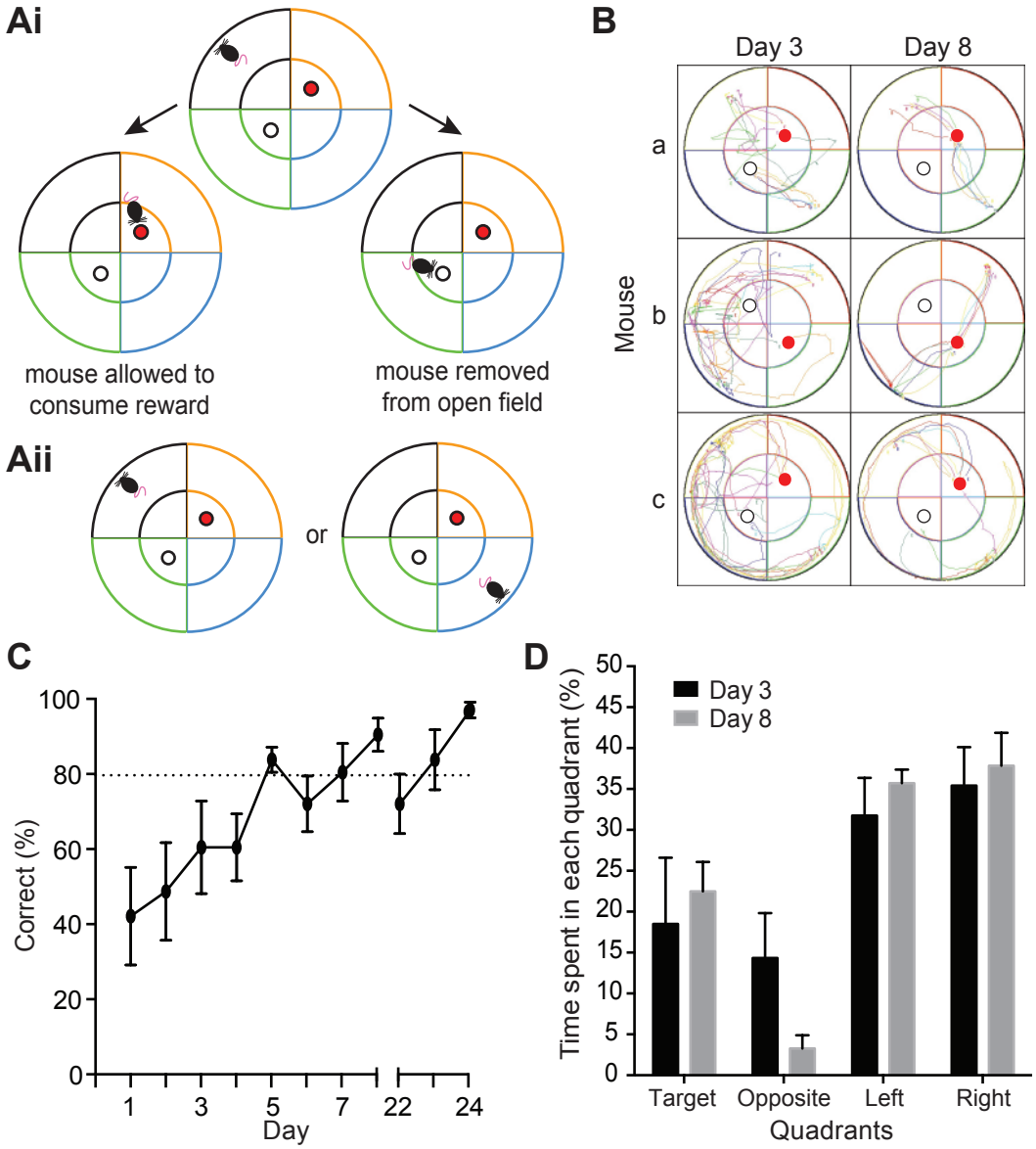
Ai
mouse allowed to consume reward
mouse removed from open field
Aii
or
B
Day 3
Day 8
Mouse
a
b
c
C
Correct (%)
100
80
60
40
20
0
1
3
5
7
22
24
Day
D
Time spent in each quadrant (%)
50
45
40
35
30
25
20
15
10
5
0
Day 3
Day 8
Target
Opposite
Left
Right
Quadrants

Figure 5.3: Place learning on an open field. (Ai) Mice were trained to find the food reward (red dot), which remained at a fixed location in relation to visual cues in the room, in an elevated open-field arena that had been divided into 8 sections. Mice were allowed to consume the reward if they approached the food well in the rewarded section. If they approached the wrong food well, mice were removed from the arena. **(Aii)** Mice started in the outer sections of the quadrants that were left or right adjacent to the rewarded section in a pseudorandom sequence. **(B)** Search paths on day 3 and day 8 for 3 representative mice. Each line represents one trial. As the mice learn the task, their paths became more directed towards the food reward. **(C)** Mice (n=6) received blocks of 10 trials a day for 11 days (eight consecutive days and their long-term memory was tested again two weeks later) and the number of correct food well choices was recorded. Their performance improved over the course of the testing period. Dashed line represents learning criterion of 80%. **(D)** Percentage of time spent in each quadrant on Day 3 and Day 8 of the testing period (quadrants: T=target, O=opposite, L=left adjacent and R=right adjacent).

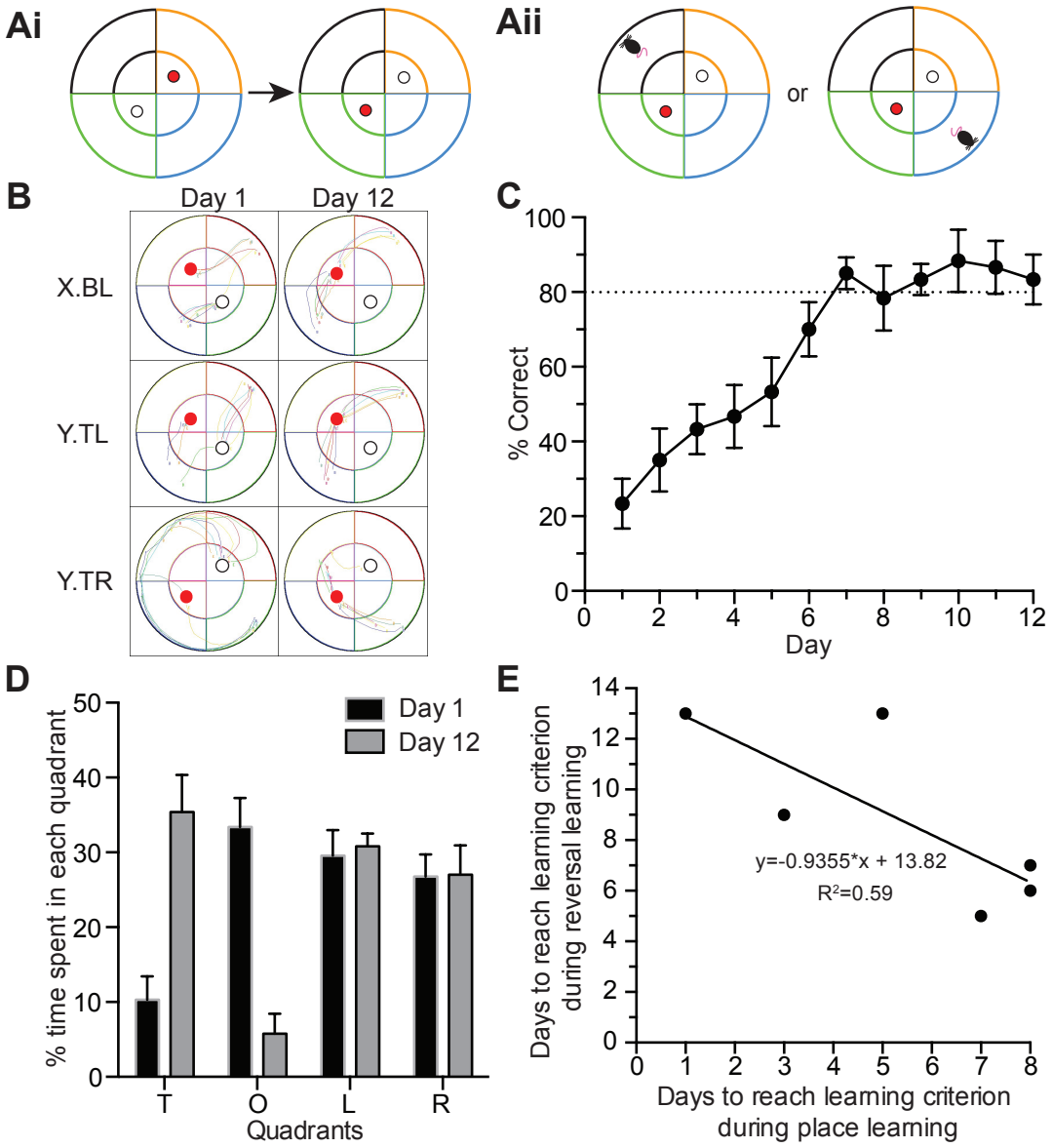
Ai
Aii
or
B
Day 1
Day 12
X.BL
Y.TL
Y.TR
C
% Correct
Day
D
Day 1
Day 12
% time spent in each quadrant
T
O
L
R
Quadrants
E
Days to reach learning criterion during reversal learning
y=-0.9355*x + 13.82
R²=0.59
Days to reach learning criterion during place learning

Figure 5.4: Reversal learning on an open field. (Ai) Mice were trained to find the food reward (red dot), which had moved to the opposite quadrant in relation to the reward location during their learning task. **(Aii)** Mice started in the outer perimeter of the quadrants, which were left or right adjacent to the rewarded section in a pseudorandom sequence, same starting places as in the learning task. **(B)** Search paths on day 1 and day 12 for 3 representative mice. Each line represents one trial. **(C)** Mice (n=6) received blocks of 10 trials a day for 12 consecutive days and the number of correct food well choices was recorded. Their performance improved over the course of the testing period, but not all reached the learning criterion of 80% (n=2, dashed line). **(D)** Average percentage of time spent in each quadrant on Day 1 and Day 12 of the testing period (quadrants: T=target, O=opposite, L=left adjacent and R=right adjacent). **(E)** Number of days required for each mouse to reach learning criterion of at least 80% correct for the remainder of the testing period during place and reversal learning. The 2 mice that did not learn the new location were listed as 13 days. It appears that the longer the mouse took to learn the reward location, the fewer days it took to unlearn the reward location and learn the new location. R^2=0.5946; p=0.0726.

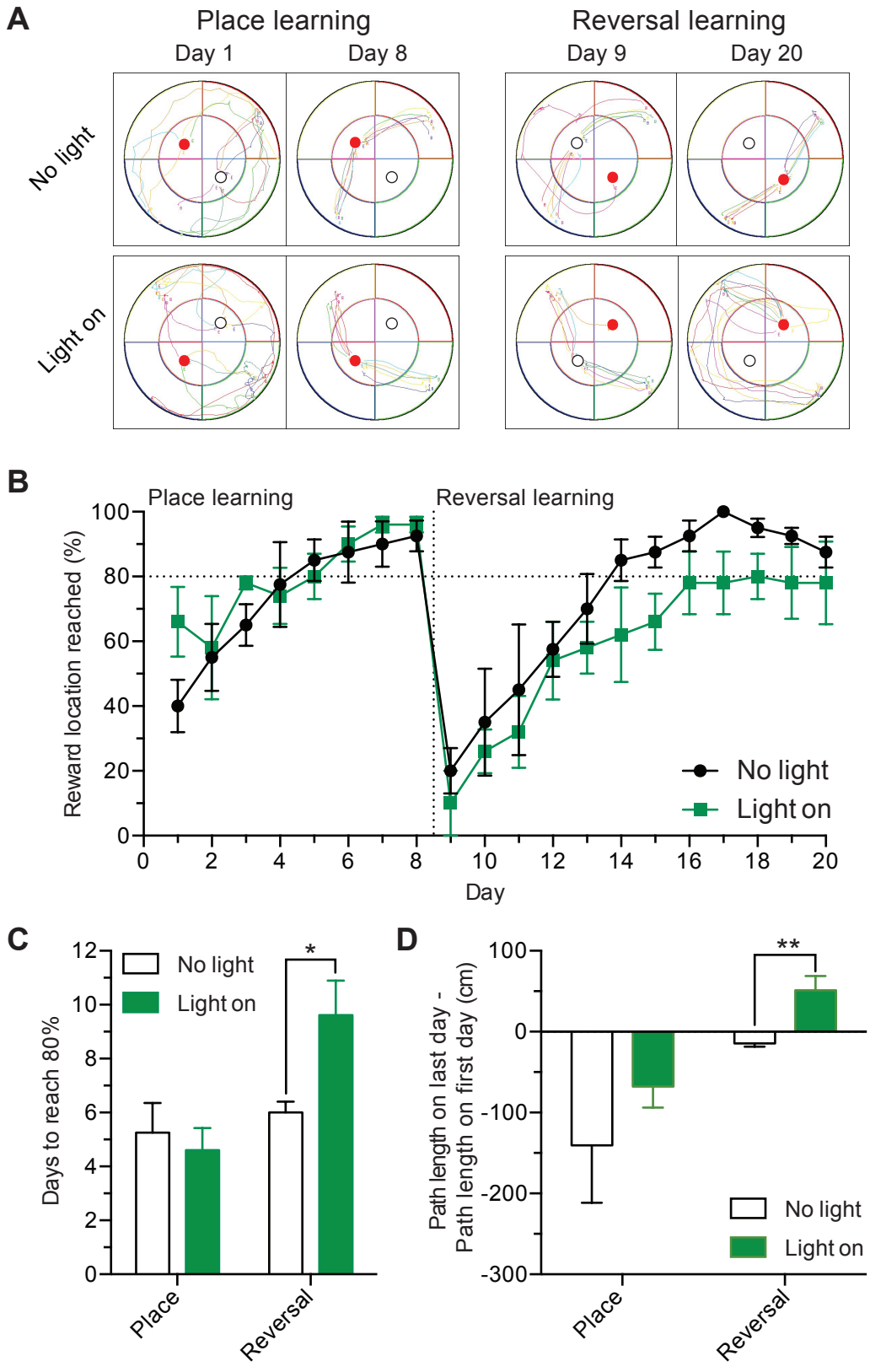
A
Place learning
Reversal learning
Day 1
Day 8
Day 9
Day 20
No light
Light on
B
Place learning
Reversal learning
Reward location reached (%)
Day
No light
Light on
C
Days to reach 80%
No light
Light on
Place
Reversal
D
Path length on last day - Path length on first day (cm)
No light
Light on
Place
Reversal

Figure 5.5: Cholinergic inactivation in ChAT-Ai40D mice yield normal long-term spatial memory, but disrupts reversal learning on the open field arena. **(A)** Search paths on the first and last day of place (left) and reversal (right) learning for a representative ChAT-Ai40D mouse with no light stimulation (top) and a ChAT-Ai40D mouse with light stimulation throughout the trial (bottom). Each line represents one trial. **(B)** Mice received blocks of 10 trials a day for 8 days during the learning period. The reward location was moved to the opposite quadrant on the ninth day when the unlearning period began. Mice received blocks of 10 trials for a further 12 days. The number of correct food well choices was recorded every day. The performances of mice receiving no light stimulation (n=4) and light stimulation throughout the trial (n=5) were similar during the place learning period but during the reversal learning phase, one of the mice in the 'light on' group did not reach the learning criterion, while all the mice that did not receive light stimulation did. Dashed line indicates the learning criterion of 80%. **(C)** The average number of days required for each group of mice to reach the learning criterion of at least 80% correct for the remainder of the testing period. Mice that did not unlearn were listed as 13 days.*$p<0.05$. **(D)** The average difference in the distance travelled between the first and last day of the place or reversal learning period for each group of mice. **$p<0.01$.

point. The difference in path lengths between the first and last day of the reversal learning period for the 'no light' group decreased by an average of –14±4 cm. In contrast, the 'light on' group travelled further distances on the last day, travelling an average of +51.2±17.6 cm further than on the first day of reversal learning (one-tailed Mann-Whitney test: p=0.008, Figure 5.5D). Together, these results indicate that while cholinergic inactivation does not significantly affect place learning, it impairs reversal learning. It appears that during reversal learning, rather than taking the most direct route to the new reward location, the 'light on' group of mice struggled to unlearn previously learnt paths and as such travel further distances to learn the new reward location.

5.3.2 Dopaminergic activation may enhance place learning

The DA system has been implicated in a range of functions including motivation, reward learning and memory. Application of DA has been demonstrated to facilitate LTP (Brzosko et al., 2015) and improve memory (Daniel et al., 1991; White and Viaud, 1991; Lange et al., 1992; Messias et al., 2016). However, the relationship between DA levels and cognitive performance can be beneficial or detrimental, depending on the basal DA levels, tasks and regional variations (Cools, 2006). Here, the role of DA activation on the OF task was examined using DAT-Ai32 mice implanted with a dual optic fibre cannulae intended to stimulate dopaminergic neurons in both the left and right VTA.

Dopaminergic activation was achieved by stimulation of ChR2 by 25 mW (light intensity at the end of the ziconia sleeve used to connect the laser patch cable to the implant), 473 nm light delivered as 50 ms-long pulses at 10 Hz frequency. These mice were divided into two groups: 'food only' group which only received food reward if they reached reward location (n=6) and 'food and light' group which received the food reward and dopaminergic activation if they reached the

correct inner quadrant (n=5). Negative littermates (WT) were also implanted as a control group and they were also divided into two groups: 'food only' (n=5) and 'food and light' (n=6).

These mice were food deprived and habituated to the OF, then I trained the mice to find the food reward located in a fixed location in relation to extra-maze cues. Unlike previous experiments performed with C57BL/6J and ChAT-Ai40D mice, mice in this cohort received blocks of 10 trials every 24-36 hours, contributing to a slower learning rate. All of the mice in both groups learnt the task in 12 blocks (Figure 5.6B), albeit at different rates. Mixed-design ANOVA with a within-subjects factor of block (1–12) and between-subjects factors of genotype (WT, DAT-Ai32) and light ('food only', 'food and light') revealed main effects of block: $F(11,204)=2.525$, $p=0.005$,, main effects of genotype: $F(1,204)=16.097$, $p<0.001$ and a significant interaction between genotype (WT/DAT-Ai32) and light stimulation: $F(1,204)=7.134$, $p=0.008$.

WT mice in the 'food only' and 'light and food' groups required 10.8 ± 1.1 and 8.7 ± 1.6 blocks to reach learning criterion (80% accuracy for the remainder of the testing period), respectively. DAT-Ai32 mice in the 'food only' and 'light and food' groups required 9.3 ± 1.7 and 4.2 ± 1.5 blocks to reach learning criterion, respectively. Two-way ANOVA of the number of blocks required to learn the task revealed main effect of light stimulation: $F(1,18)=5.58$, $p=0.03$, but no main effect of genotype: $F(1,18)=3.72$, $p=0.07$; nor interaction between light and genotype: $F(1,18)=0.9511$, $p=0.34$; Figure 5.6). These preliminary data reveal a trend that indicates that dopaminergic activation may enhance place learning.

A potential criticism of the results presented is that the 'food and light' DAT-Ai32 group, which appear to learn the task the fastest, also displayed superior performance from the first block of learning. Therefore, it would not be surprising that they would be the first group to reach learning criterion. Further analysis

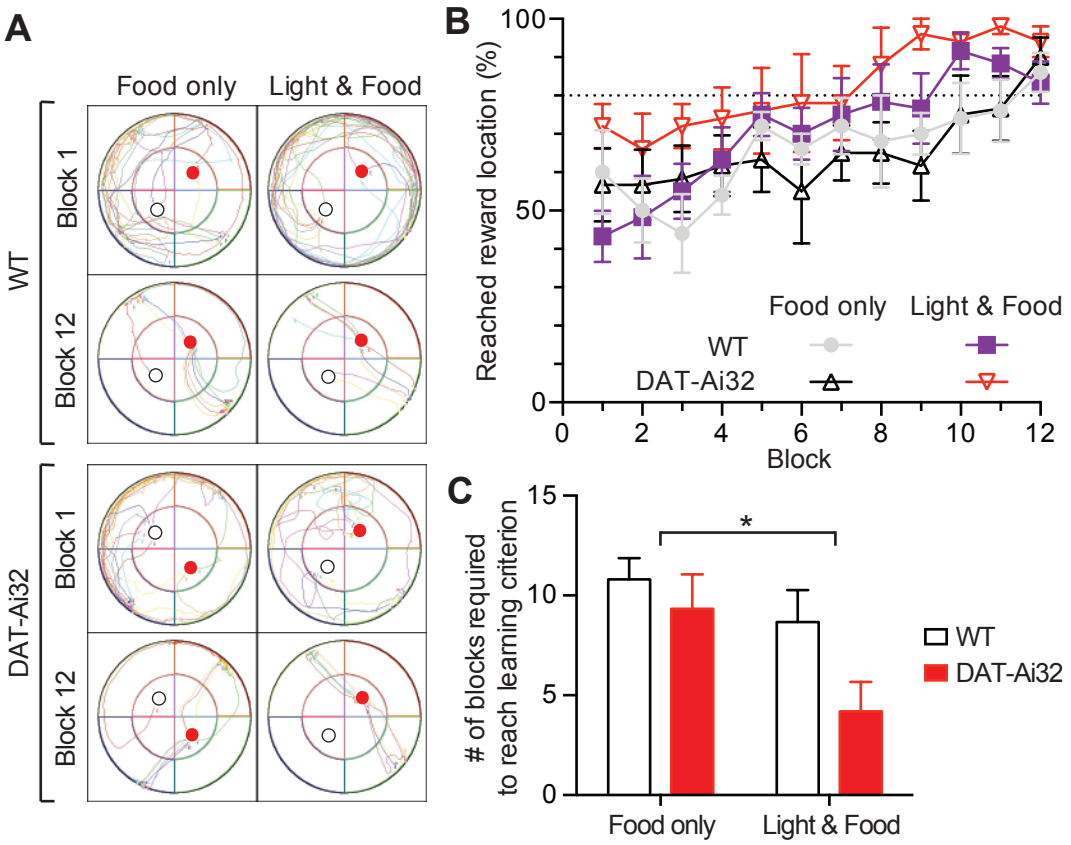

Figure 5.6: Activation of dopaminergic neurons appears to improve place learning. (A) Search paths during block 1 and 12 for a representative mouse from each group. Each line represents one trial. **(B)** Mice received 12 blocks of 10 trials and the number of correct food well choices was recorded. The performance of wild-type mice receiving 'food only' (n=5) and 'food and light' stimulation (n=6) as well as DAT-Ai32 mice receiving 'food only' (n=6) and 'food and light' stimulation (n=5) improved over the course of the testing period. Dashed line represents the learning criterion of 80% correct for the remainder of the testing period. **(C)** The average number of blocks required for each group of mice to reach the learning criterion.

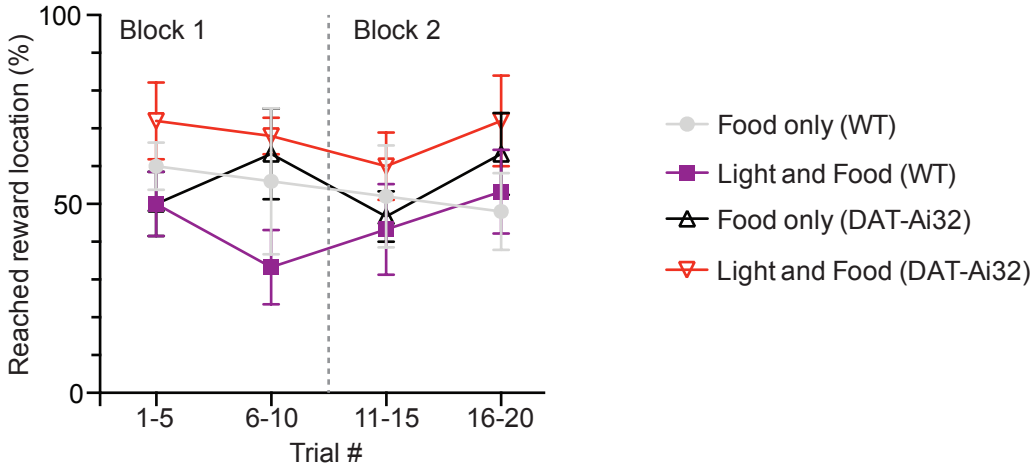

Figure 5.7: There is large variation in task performance. The average performance for each group of mice over the first 20 trials (Block 1 and Block 2).

of the performance on the first twenty trials (Figure 5.7) suggest that the 'food and light' DAT-Ai32 group did not appear to learn more quickly on the first block (i.e. learnt location within the first 10 trials) with declining performance following the first 5 trials. There is large variation on performance due to a low number of subjects. Mice were randomly assigned to groups based on genotype and thus this inter-individual differences should in theory disappear with a larger cohort of mice.

5.3.3 Dopaminergic activation may have an effect on reversal learning

After 12 blocks of place learning, the reward was moved to the opposite quadrant to test performance on reversal learning. Not all of the mice learnt the task but there was no difference in their rate of learning (Figure 5.8B). Analysis of the performance on the last block of testing (Figure 5.8D) showed that the 'food and light' DAT-Ai32 group performed the worst, with only one out of five mice meeting the learning criterion (>80%) and reaching the reward location at an average of 62±7% compared to 82±5%, 80±8% and 78±12% for the 'food only' DAT-Ai32, 'food only' WT and 'food and light' WT groups,

respectively. Notably, only one mouse from each of the other groups did not meet the learning criterion on the last block of reversal learning. The difference in the performance did however not reach significance. These preliminary findings suggest a trend where dopaminergic activation may impair reversal learning.

5.3.4 Transient dopaminergic activation alone is not sufficient to induce learning of a goal location

Rewards or punishments can alter DA signalling and VTA dopaminergic neurons are known to spike either phasically or tonically in various behavioural conditions. Recent optogenetic studies revealed that selective phasic stimulation of VTA dopmainergic neurons is sufficient to drive behavioural conditioning (Tsai et al., 2009; Chang et al., 2015; Witten et al., 2011). Whether transient DA signal also drives reward goal-directed learning in the hippocampus remains uncertain. Therefore, I sought to determine whether DA activation alone at the reward location is sufficient to induce learning in a long-term spatial memory task.

I implanted DAT-Ai32 mice (n=7) with dual optic fibre cannulae in the VTA and trained them on the OF task. While the food reward wells remained present on the maze, they were empty. Instead of receiving food reward, these mice were 'rewarded' with light stimulation (25 mW, 50 ms-long pulses at 10 Hz frequency) that was delivered as soon as they enter their pre-designated goal zone (one of four inner quadrants). Light stimulation continued for 20 seconds (i.e. the duration often required by mice to finish food reward), or for as long as the mice remained in the goal area, whichever elapsed first. Negative litter-mates (WT; n=5) as control were also implanted and received the same light stimulation if they reached the goal zone. Once on the OF, mice were removed if they remained stationary for 1 minute, or they entered the inner

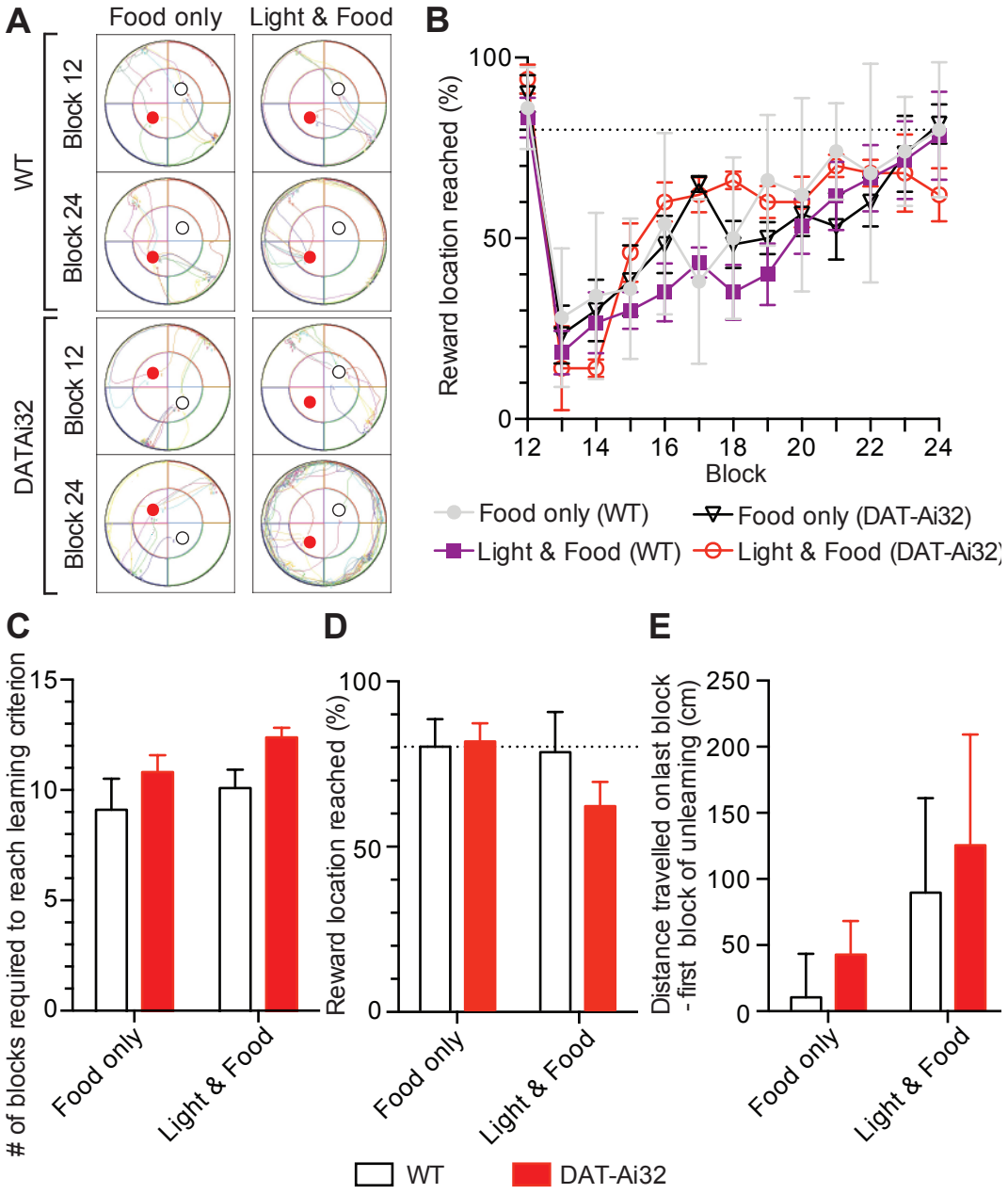

Figure 5.8: Activation of dopaminergic neurons appears to impair reversal learning. (A) Search paths during block 12 and 24 for a representative mouse from each group. Each line represents one trial. **(B)** Mice received 12 blocks of 10 trials and the number of correct food well choices was recorded. The performance of wild-type mice receiving food reward (n=5) and light stimulation and food (n=6) as well as heterozygous DATAi32 mice receiving food reward (n=6) and light stimulation and food (n=5) improved over the course of the testing period, but not all reached the learning criterion of 80% (dashed line). **(C)** The average number of blocks required for each group of mice to reach the learning criterion of at least 80% correct for the remainder of the testing period. Mice that did not unlearn were listed as 13 blocks. **(D)** The average performance on the last block of the testing period. **(E)** The average difference in path lengths between the first and last block of reversal learning.

quadrant opposite to the goal location, or if they did not reach the goal area within 2 minutes, whichever occurred first.

None of the mice learnt the task but interestingly, DAT-Ai32 mice remained motivated to move and find the goal location, on average travelling significantly longer distances (DAT-Ai32: 403.8±37.96 cm vs. WT: 218.9±27.97 cm; two-tailed Mann Whitney test: p=0.0002) and reaching the reward location in 25.71±10.2% of trials compared to 0% in WT mice on Day 12 of testing (Figure 5.9). WT mice were consistently removed from the maze after the third block of trials due to lack of movement. This data suggest a role for DA in motivation.

5.4 Verification of implant location

In order to attribute the results to the effects of cholinergic activation/inactivation or dopaminergic activation, it was important to confirm that the implants were in a position that was able to illuminate the cholinergic or dopaminergic neurons in the MS or VTA respectively. Therefore, after behavioural testing, all of the mice tested were perfused and their brains were fixed, sliced and mounted to allow verification of opsin expression and implant location.

To verify opsin expression, slices containing the MS or VTA were viewed under a fluorescent microscope or a confocal microscope. To determine implant positions, I viewed slices that contained the implant tract under a bright-field microscope and noted placements of implants as defined by the deepest extent of the implant. All of them were in a position that indicated that they would illuminate the desired brain region. Moreover, there was no indication that the position of the implants varied systematically between the experimental groups

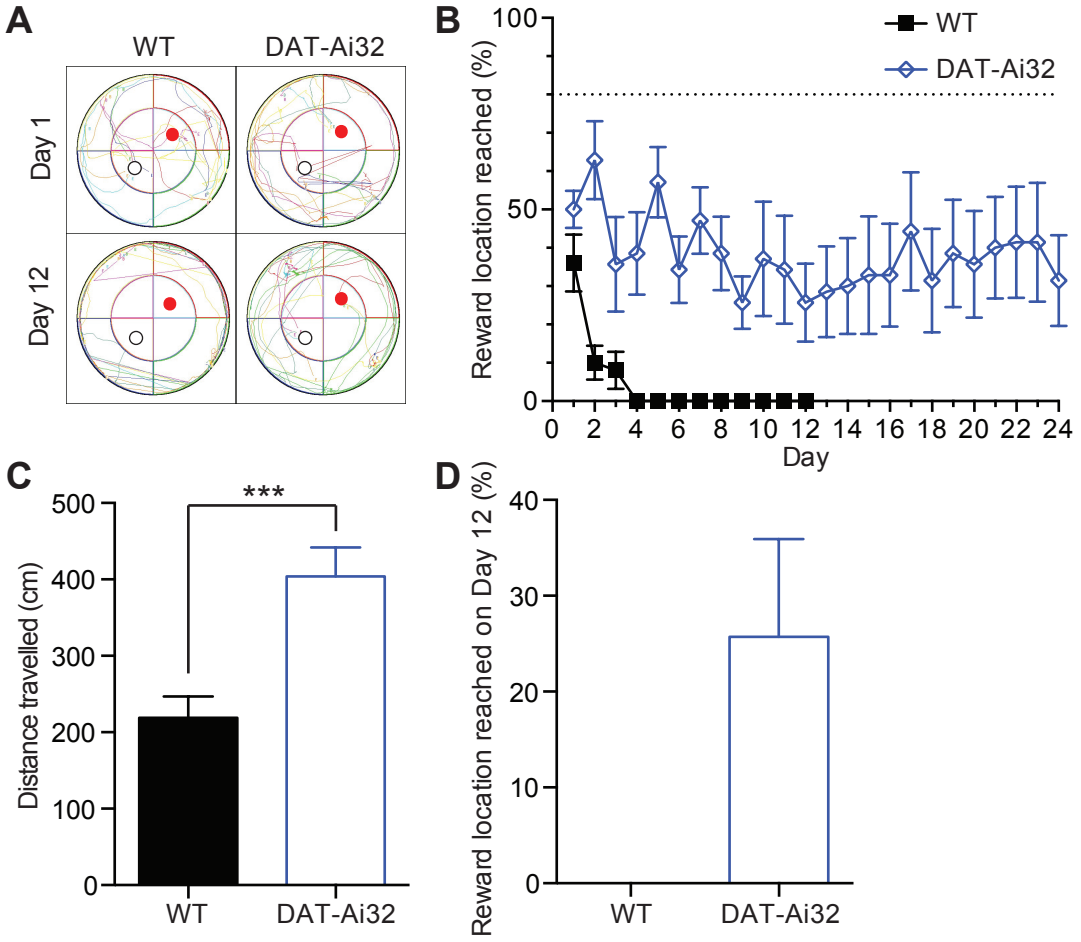

Figure 5.9: Activation of VTA dopaminergic neurons only is not enough for mice to develop a place preference. (A) Search paths on block 1 and 12 for a representative mouse from each group. Each line represents one trial. **(B)** The place learning curve. DAT-Ai32 that received light stimulation only (n=7) did not reach the learning criterion (80%, dashed line) but continued exploring the OF. In contrast, WT mice (n=5) receiving light stimulation stopped exploring the OF all together. **(C)** Mean distance travelled for WT and DAT-Ai32 mice. **(D)** Performance on day 12 of place learning for each group of mice. Error bars indicate S.E.M. ***$p<0.001$

(Figure 5.10).

While how far the light spreads and its fall off through tissue can be estimated, little is known about the power of light required to activate/inhibit neurons with different excitatory drives. Furthermore, how a given wavelength of light and its interaction with various levels of opsin expression over various distances presents another source of variability in the extent of excitation/inhibition attained. A comprehensive characterisation of such effects was beyond the scope of this thesis. As a result, as there were limited satisfactory indicators to determine fair exclusions, two broad guidelines were used to avoid introducing any bias into the analysis. First, expression must be present in the MS/VTA and hippocampus; second, the tip of the fibre-optic implant was less than 1.5 mm from the top of the MS or VTA. All mice met these criteria and, as such, no exclusions were made.

5.5 Discussion

Three principal findings emerged from the work presented in this chapter. First, cholinergic activation during reward slows learning on the appetitive Y-maze task. I then created an appetitively-motivated OF task that can be used to study place and reversal learning. Using this maze, I found that cholinergic inactivation does not affect place learning but instead, impairs reversal learning. Lastly, while transient dopaminergic activation alone is not sufficient to induce learning of a goal location, dopaminergic activation along with food reward appears to enhance place learning and may impair reversal learning. However, it is important to note the caveat that given the effects of optical stimulation observed in Figure 3.14, in which optical stimulation in the VTA of DAT-Ai32 mice reduced MUA activity, the effects observed in experiments involving DAT-Ai32 mice could be due to inactivation, and not due to the presumed dopaminergic

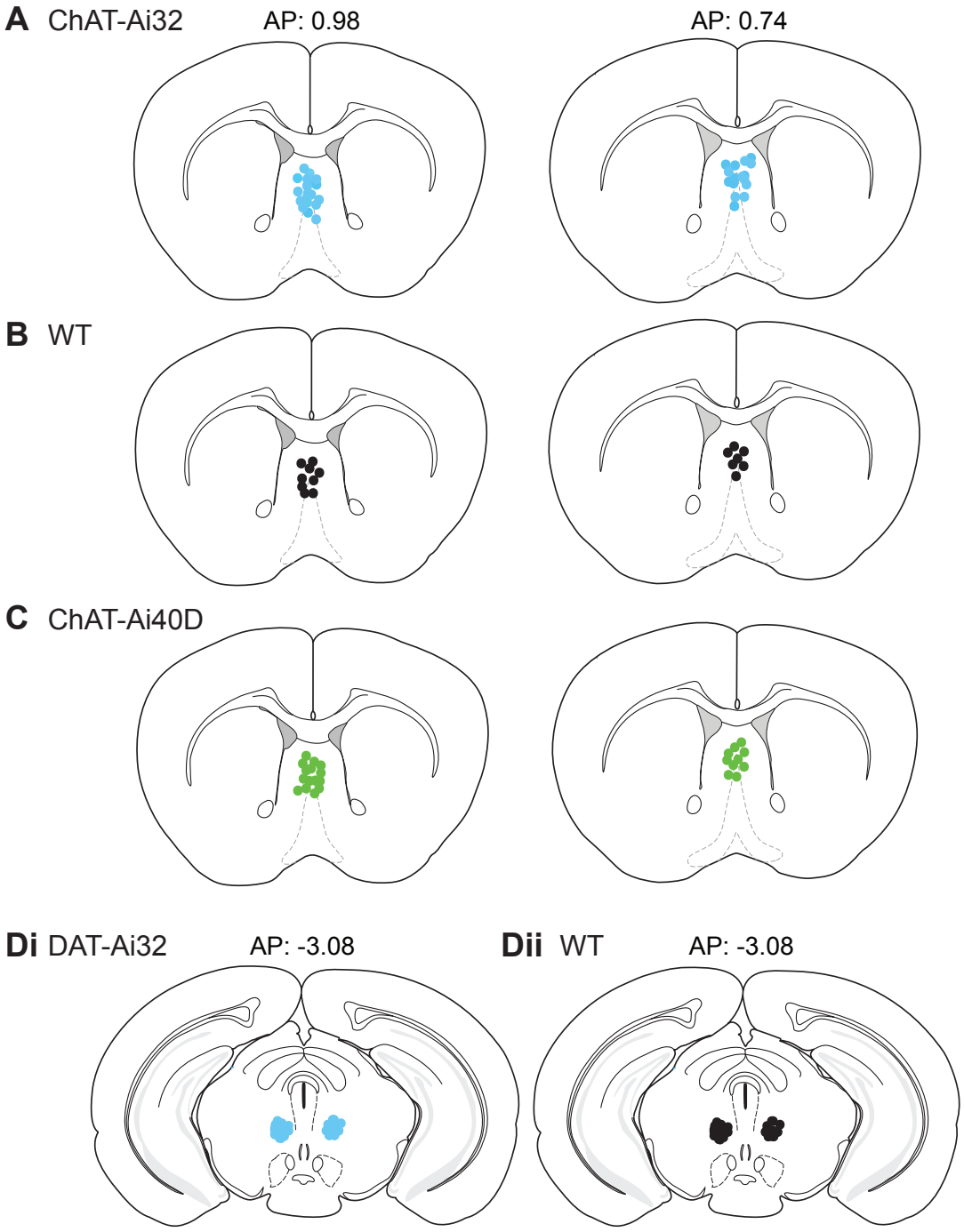

Figure 5.10: Placements of the fibre optic implants were verfied post-testing. The approximate locations of the fibre-optic tip for each ChAT-Ai32 mouse (n=36; **A**), wild-type litter-mate (n=17; **B**), ChAT-Ai40D mouse (n=26; **C**), DAT-Ai32 mouse (n=18; **Di**) and their wild-type litter-mate (n=16; **Dii**) used in behavioural testing.

activation.

5.5.1 Cholinergic activation during different phases of long-term spatial memory

ACh has been postulated to have a biphasic role in memory formation. During exploration, levels of ACh are high and promote theta and gamma oscillations that thereby facilitate memory encoding. During subsequent rest, ACh levels are low, enabling SWRs, which promote memory consolidation. In a bid to examine the differential effects of ACh on various phases of memory formation, I optogenetically activated cholinergic neurons in freely moving mice during different phases of the appetitive Y-maze task. The appetitive Y-maze task has been used extensively to study a variety of hippocampus-dependent spatial learning processes (Bannerman et al., 2012; Brown-borg et al., 2010; Shipton et al., 2014; von Engelhardt et al., 2008). The task can be split into two phases, exploration, during which memory encoding occurs, and reward, during which memory consolidation takes place. I found that cholinergic activation during reward slows learning.

Combined with the results observed in chapter 3, my working hypothesis is that suppression of SWRs via the activation of the cholinergic system during reward consummatory behaviours slows learning. Several studies have demonstrated the importance of SWRs during memory consolidation. Disruption of awake sharp waves (during consummatory behaviour) with electrical stimulation results in learning and spatial memory performance deficits (Girardeau et al., 2009; Ego-Stengel and Wilson, 2010; Jadhav et al., 2012).

During exploration, a behaviour associated with theta and gamma oscillations, ACh activation had little effect on learning performance. Levels of ACh are already high (Fadda et al., 1996; Giovannini et al., 1998; Fadda et al., 2000;

Hironaka et al., 2001; Fadel, 2011) and additional ACh did not augment or improve performance compared to other ChAT-Ai32 mice that did not receive any cholinergic activation throughout the task.

This finding highlights the importance of the temporal precision in neuromodulation and that tonic increase of ACh is not always beneficial. This differential effects of ACh at different phases of learning is of particular interest. AChE inhibitors that maintain ACh level by preventing its breakdown are one of the main treatments for AD (Colović et al., 2013). Unlike optogenetics, which artificially force neurons to spike, the mode of action of AChE inhibitors temporally links to the physiological release of ACh. However, exactly how long ACh remains extracellularly with administration of AChE inhibitors and whether this significantly impairs hippocampal replay in the awake state is uncertain.

5.5.2 Acetylcholine and reversal learning

In order to understand the effects of cholinergic inactivation on spatial learning, I optogenetically inactivated cholinergic neurons in freely moving mice during a novel, appetitively-motivated OF task. Cholinergic inactivation impairs reversal learning but does not affect spatial navigation and motivation. Mice with cholinergic inactivation throughout the trial actively search for the reward and consume it as soon as they find it. Rather, they appear to be impaired in their ability to adapt their behaviour to environmental changes, as demonstrated by their deficit in reversal learning.

There has been accumulating evidence that ACh plays an important role in reversal learning. Ibotenic acid lesions of the basal forebrain, which result in decreased ChAT activity, impair reversal learning in marmosets (Ridley et al., 1985). On the other side, ACh activity has been shown to enhance reversal learning; selective activation of M_1 receptor enhances activity of medial pre-

frontal cortical neurons and restores reversal learning deficit observed in a transgenic AD mouse model (Shirey et al., 2009). Moreover, modulation of ACh efflux within the dorsomedial striatum during spatial reversal learning suggests that striatal cholinergic neurons are activated by novel stimuli and appears to enhance the learning of a new spatial location when reward values of two locations are reversed (Ragozzino et al., 2002; Tzavos et al., 2004; Ragozzino and Choi, 2004), thereby enabling behavioural flexibility. My findings concur with these findings where lack of ACh by cholinergic inactivation in ChAT-Ai40D mice impairs reversal learning.

At the level of cellular networks, ACh has been shown to facilitate LTD which has been hypothesised to be critical for behavioural flexibility. Interfering with NMDAR-dependent form of LTD impeded reversal learning (Nicholls et al., 2008). Similarly, I suggest that the deficit observed in reversal learning by cholinergic inactivation may impede LTD required to weaken the previously encoded memory traces as new information is acquired, which manifested as a deficit in behavioural flexibility.

5.5.3 Dopamine and reward learning

DA neurons have long been implicated in reward learning, they encode reward prediction errors (violations in expected outcomes; Schultz, 2016) and can be manipulated to facilitate and disrupt different forms of learning (Tsai et al., 2009; Adamantidis et al., 2011; Steinberg and Janak, 2013).

In an effort to further understand the reinforcing properties of dopaminergic neurons, I optogenetically activated dopaminergic neurons in freely moving mice during the OF task. Other studies have shown that dopaminergic stimulation biases choice of stimuli (Tsai et al., 2009; Adamantidis et al., 2011; Kim et al., 2012; Kempadoo et al., 2016; Stauffer et al., 2016); subjects pref-

erentially choose the place/stimuli associated with dopaminergic stimulation. Consistent with other studies, I showed that there is a trend that transient optical stimulation of dopaminergic neurons enhance learning of a food reward-associated goal location in food-restricted DAT-Ai32 mice when compared to mice receiving the food reward alone and in WT mice. Interestingly, I found that optogenetic activation of dopaminergic neurons alone (i.e. in the absence of food rewards) was not effective in driving learning of the goal location. These findings were similar to those presented by Adamantidis et al. (2011) with mice performing a self-stimulation study where they found that while dopaminergic and food reward biases the choice in lever selection, dopaminergic stimulation alone did not. Multiple possible interpretations may explain these observations.

First, one of the initial studies in reward processing identified the medial forebrain bundle, lateral hypothalamus, VTA and several brainstem nuclei as "brain stimulation reward" sites since animals would repeatedly self-stimulate electrically fibres and cells within these brain areas (Olds and Milner, 1954; Wise, 2004; Carlezon Jr and Chartoff, 2007). This electric stimulation activates a heterogeneous population of cells and fibres including glutamatergic (Wise and Bozarth, 1984; Geisler et al., 2007) and cholinergic fibres (Blaha et al., 1996; Omelchenko and Sesack, 2005) that eventually excite dopaminergic neurons and induce DA release (Gratton et al., 1988; Wise, 2004). While the outcome of this self-stimulation electrical stimulation converges towards dopaminergic activation and DA efflux, co-activation of parallel excitatory and inhibitory fibres is also likely to contribute to this reward behaviour. Therefore, concurrent activation of additional nondopaminergic circuits and neuromodulators, such as those related to food-seeking behaviour may be needed for learning, as suggested by my results. Particularly, it is well known that food reward activates both dopaminergic and nondopaminergic neurons in several brain regions, such as the orexin/hypocretin system in the lateral hypothelamus that may indirectly potentiate the acquisition of place learning (Harris and Aston-Jones, 2006).

Second, whether optogenetically induced DA release mimics that in rewarded naive animals is unclear. Dopaminergic neurons in the VTA respond to unpredicted reward with bursts of phasic activity (i.e. brief series of spikes). Indeed, in other studies that induced place/choice preference by phasic stimulation, they used high frequency light delivery parameters such as bursts of 50-Hz 25 light flash trains, 15-ms long flashes delivered with a periodicity of 1 minute in Tsai et al. (2009); 25-Hz (5 ms pulse width; 20 pulses) in Adamantidis et al. (2011), a single transient 200-ms long pulse in Kim et al. (2012) or 10-ms long pulses delivered with an inter-pulse interval of 15–30 ms in Stauffer et al. (2016). Tsai et al. (2009) further explored the difference between phasic and tonic stimulation and found that while phasic stimulation was sufficient for behavioural conditioning, tonic stimulation was not. It is unlikely that dopaminergic neurons respond with such uniform frequency, nevertheless, in comparison, the stimulation protocol used in my experiment may more mimic tonic stimulation. The stimulation frequency was quite slow with constant 10-Hz 50-ms-long pulses which may be more equivalent to tonic stimulation that has been suggested to be more important for motivational drive. This was best illustrated in the experiment with no food reward; DAT-Ai32 mice receiving dopaminergic activation continued to search for reward while WT mice did not. Nevertheless, given the aim of the experiment, a faster stimulation protocol may be more appropriate in hindsight. This 10-Hz, 50-ms-long pulses stimulation protocol was the same protocol used for cholinergic activation in ChAT-Ai32 mice in an effort to fairly compare the two systems. However, perhaps it would have been better to place more importance on natural DA neuron firing rates. It would be interesting to repeat the same experiment using a burst firing stimulation protocol. It would also be crucial to measure the release of DA in the hippocampus by performing amperometry.

Third, optical dopaminergic activation in my experiment was associated and time-locked with entry to the reward location area, which, in relation to the prediction error hypothesis, may have led to a non-optimal DA encoding of re-

ward signal and was thus insufficient to induce goal-directed learning. Indeed, in the study published by Tsai et al. (2009), mice developed place preference after receiving 30 minutes of phasic photoactivation for a number of days. In my experiment, mice often moved away from the goal area within 10 seconds and this temporal encoding constraint in my experiment may account for the small effects of dopaminergic activation.

5.5.3.1 Reversal learning

After place learning, I also tested whether optogenetic activation of DA neurons may affect reversal learning on the OF task. I found a trend that indicates optogenetic activation of dopaminergic neurons may impair reversal learning.

However, in addition to some of the limitations outlined above, this reversal learning experiment may be biased by inter-group learning variability during place learning. As observed in the pilot experiment (Figure 5.4E), there may be a negative correlation between the number of days required for place and reversal learning. It seems that the faster a mouse learns the goal location, the longer it requires to learn the new location. This is not surprising as the more the previous rewarded location is repeatedly visited, the more the memory is reinforced in the networks. As the 'food and light' DAT-Ai32 group appears to learn more quickly than the other groups, it is thus perhaps not so surprising that this group took the longest to learn the new location. A further experiment where place learning is unaffected may be required to truly test the effects of transient DA during reversal learning.

Nevertheless, Morice et al. (2007) showed that knockout mice for the dopamine transporter (DAT-ko), a genetic model of constitutive hyperdopaminergia, also display impaired reversal learning in the Morris water maze. In addition, electrophysiology experiments in these DAT-ko reveal selective impairment in

hippocampal LTD (Morice et al., 2007); both of these deficits can be reversed by haloperidol. These findings suggest that the deficit in LTD manifests as impaired adaptation to environmental changes. Interestingly, these DAT-ko mice also exhibit enhanced LTP and normal spatial reference memory. Similarly, Boulougouris et al. (2009) show that systemic administration of the D2/D3 receptor agonist quinpirole significantly impairs reversal learning in a spatial discrimination task. While co-administration of quinpirole and raclopride (a D2/D3 receptor antagonist) reverses the quinpirole-induced deficit, combined administration of nafadotride (a selective D3 antagonist) and quinpirole did not, suggesting a crucial role for D2 receptor in behavioural flexibility in the face of environmental changes (Ridley et al., 1981; Ragozzino, 2002; Kruzich and Grandy, 2004; Floresco et al., 2006; Izquierdo et al., 2006; Lee et al., 2007; Boulougouris et al., 2009).

5.5.4 Perspective

The findings presented in this chapter reveal an interesting dissociation of the effects of cholinergic activation between memory encoding and consolidation. This is consistent with the conclusion that ACh has a biphasic role in hippocampal memory formation and that precise timing of neuromodulator action is critical for optimal learning and memory performance. I have hypothesised that a high level of ACh during exploration facilitates theta and gamma oscillations enabling memory encoding. ACh also enhances the induction of LTD (Brzosko et al., 2017), which facilitates acquisition of novel spatial information. I observed that in ChAT-Ai40D mice, cholinergic inactivation impaired reversal learning, further supporting the assumption that impaired ACh response, believed to act via a LTD deficit, impairs the learning of novel information.

During memory consolidation, low levels of ACh allow for SWRs and LTP to occur. Cholinergic activation during memory consolidation suppresses SWRs

and thereby impairs learning. A demonstration of whether this explanation does underpin the impairment observed requires concurrent hippocampal LFP recordings while the mice perform the behavioural task. In an effort to record hippocampal LFP in freely moving mice, I implanted two ChAT-Ai32 mice with chronic electrodes secured to the skull using dental cement (Figure 3.9). While a couple of recordings were made, the "signal-to-noise" ratio was too high for chronic recordings during fast movements. A more stable, chronic probe, such as a silicon probe, would be needed.

In this chapter, I also explored the effects of transient DA on place and reversal learning. The results support a role for DA in reinforcement learning and motivation. DA has been shown to bias plasticity to potentiation (Brzosko et al., 2015) and enhance protein synthesis required for late LTP (Huang and Kandel, 1995). This is complimentary to the role of ACh in reward-based navigation. While ACh enhances LTD (Brzosko et al., 2017) and the learning of novel information during memory encoding, DA enhances LTP (Brzosko et al., 2015) and reinforces preceding action choice and improves motivation. Together, ACh and DA support complementary processes that allow for effective learning and adaptation to changing environments.

Chapter 6

General discussion and conclusion

6.1 Main findings

The key findings of this thesis are:

1. Optogenetic activation of the cholinergic system suppresses slow oscillations and enhances theta and slow gamma oscillations in urethane-anaesthetised mice. These effects depend on mAChRs as administration of scopolamine blocks these effects. Conversely, cholinergic inactivation enhances SO. Dopaminergic activation suppresses hippocampal gamma oscillations without significantly affecting slow or theta oscillations.

2. Cholinergic modulation of spatial WM has variable effects. Cholinergic activation during Phase 1 of the SLR task appears to change object preference. On the other hand, cholinergic inactivation may impair SLR task performance. However, the results of these experiments were difficult to interpret due to suboptimal experimental design and large variation in the data.

3. Cholinergic activation during reward slows learning on the appetitive Y-maze task, indicating that an increase in ACh levels during memory consolidation could impair performance in a long-term spatial memory task. Furthermore, cholinergic inactivation does not affect place learning but instead impairs reversal learning, suggesting an important role for the cholinergic system in cognitive flexibility.

4. Transient dopaminergic activation alone is not sufficient to induce learning of a goal location but dopaminergic activation in addition to food reward appear to enhance place learning and may impair reversal learning.

In the following sections, I will briefly discuss the validity of these main findings and suggest how they can be drawn together to contribute towards our understanding of neuromodulation in hippocampal learning and memory processes.

6.2 Optogenetics as a technique to probe learning and memory processes

The ability to activate and inactivate neurons has been fundamental to investigate the cellular networks and mechanisms that underlie disease and brain function. Activation can be achieved by electrical stimulation but this method recruits fibers indiscriminately and, as such, several cell types and any resulting neuromodulatory output may contribute to the effects observed. Application of agonists or antagonists has also been used to probe neuromodulatory functions but while this approach solves the problem of cell-type specificity, it lacks temporal precision. Some other methods used to probe neuromodulatory functions include genetic knockout animal models, lesion analysis, ablation of cell types in specific brain regions using neurotoxins or immunotoxins and recently, chemogenetics. While these manipulations can be informative and offer clues regarding the action or mandatory components of a circuit, they do not directly probe which brain regions or mechanisms are engaged in intact systems during brain processes and also lack temporal resolution. Therefore, in this thesis, I used optogenetics, a technique that aims to circumvent some of the complications that arise from non-specific modulation or chronic modifications of neuromodulatory system and furthermore, enable new questions to be investigated.

Optogenetic tools allow regional- and cell type-specific activation or silencing of neurons on a millisecond time-scale and, as such, offer a precise method to directly interrogate the involvement of the cholinergic or dopaminergic systems in a wide range of processes on a network and behavioural level. In particular, it allows the cholinergic or dopaminergic system to be isolated, and its effects interrogated in an intact system in real-time.

The cell-type specificity was conferred throughout the brain using transgenic mice. While this method allows the expression of opsins to be targeted to one

cell-type, the opsins are present in most of the cholinergic or dopaminergic populations throughout the brain. The MS or VTA was targeted via the placement of the optic fibre, however, the light could spread and the effects observed may not solely depend on the cholinergic or dopaminergic neurons in the MS or VTA. To more specifically target a particular brain region, another approach would be to inject viral constructs containing Cre-dependent ChR2 or ArchT into the particular brain region (e.g. MS/vDB) of transgenic mice and thereby more limit the expression of the constructs.

A distinct advantage of optogenetics over the present pharmacological techniques is the ability to reversibly activate and silence neurons at a rapid rate. For example, the muscarinic antagonist scoplamine can take up to 10 minutes to achieve an effect that last 100-120 minutes (Thomsen, 2014). The reversibility and rapidity of optogenetics are particularly useful for investigating the specific systems *in vivo* and study their roles in behaving animals. Here, I have used optogenetics to activate or silence the cholinergic or dopaminergic system *in vivo* and the acute and rapid nature of this manipulation meant that it was possible to more precisely dissociate the differential effects of the neuromodulatory systems on different phases of learning and memory processes. However, it is important to emphasise that following the activation of cholinergic or dopaminergic neurons, it is possible that the neuromodulators released remain extracellularly following the termination of light stimulation. The action of these chemical messengers is slow (Falkenburger et al., 2010); this may be best demonstrated in Figure 3.4 where the effects of the light stimulation began after the onset of light stimulation and continued after the light stimulation has ended. It may also explain why the work presented here is not in complete agreement with some of the findings in literature (e.g. Everitt and Robbins (1997) have suggested that the septo-hippocampal cholinergic projection does not modulate spatial processing that takes place in the hippocampus to any significant degree). Nevertheless, optogenetics is a powerful tool to probe the functions of neuromodulatory systems that are involved in learning and

memory processes and how they may be affected in disease.

6.3 The effects observed may not be solely due to modulation of hippocampal processes

When evaluating the findings presented in this thesis, it is important to add the caveat that the effects of cholinergic and dopaminergic neurons observed may not be solely due to modulation of hippocampal processes. The fibre optic for ChR2 or ArchT optical stimulation was placed above the MS or VTA with the aim of directly stimulating or inhibiting cholinergic neurons in the MS or dopaminergic neurons in the VTA. As MS cholinergic neurons and VTA dopaminergic neurons do not solely project to the hippocampus, modulating these neurons could affect processes in other brain regions and their downstream pathways.

For cholinergic modulation , the fibre optic was placed directly above the MS of ChAT-Ai32 or ChAT-Ai40D mice (Figure 5.10A, C). Apart from the hippocampus, cholinergic neurons in the MS also project to the medial entorhinal cortex, olfactory bulb, prefrontal cortex, somatosensory cortex and subcortical regions (Wu et al., 2014; Unal et al., 2015; Li et al., 2017). For dominergic activation, the fibre optic was placed above the VTA of DAT-Ai32 mice (Figure 5.10D). In addition to the hippocampus complex, dopaminergic neurons in the VTA project to the nucleus accumbens and olfactory tubercle of the ventral striatum, prefrontal cortex, lateral septum, the bed of nucleus stria terminalis, amygdala, anterior cingulate cortex and lateral hypothalamus (Perogamvros and Schwartz, 2012; Morales and Margolis, 2017). Dopaminergic projections from the VTA to the hippocampus is rather sparse (Lisman and Grace, 2005; Bethus et al., 2010; McNamara et al., 2014; Rosen et al., 2015) and given the important role of nucleus accumbens in the cognitive processing of motivation, reward and

aversion (Ikemoto, 2007; Saddoris et al., 2015; Wenzel et al., 2015), it would not be surprising if the dopaminergic effects observed in Figures 5.6, 5.8 and 5.9 were actually mediated via the inputs to the nucleus accumbens. Therefore, to verify that the effects observed are mediated via hippocampal processes, placing the fibre optic, and thereby activate cholinergic or dopaminergic terminals in the hippocampus, is required.

6.4 Cholinergic modulation of hippocampal learning and memory processes

Dissecting the neural substrates that underlie learning and memory processes is critical for our understanding of cognition and how they fail in disease. While much work has been done with lesions and ablation of cholinergic neurons or brain regions, these techniques lack spatio-temporal resolution and how subtle neuromodulatory signals can affect learning and memory processes is poorly understood. To study these processes in a temporally precise manner, advancement in optogenetics has allowed the direct interrogation of the effects of neuromodulatory systems on various learning and memory processes.

6.4.1 Theta and gamma oscillations

On a cellular network level, cholinergic activation was shown to enhance theta and gamma oscillations (Figures 3.4, 3.5 and 3.8), which are thought to be important for memory encoding (Weiss et al., 2000; Osipova et al., 2006; Hasselmo and Stern, 2014). Theta and gamma oscillations are associated with periods of learning and increased power in the theta and gamma band has been shown to be predictive of successful encoding of declarative memories (Osipova et al., 2006). The physiological role of theta and gamma oscillations in memory encoding is thought to be two-fold: neuronal synchronisation and

synaptic plasticity.

Firstly, the increase in theta and gamma power during cholinergic activation most probably reflects enhanced neuronal synchronisation. The hippocampus receives multimodal sensory inputs from multiple brain areas and synchronised activity in the hippocampus is likely to result in a stronger drive to downstream areas directly involved in memory encoding (Salinas and Sejnowski, 2001). Furthermore, hippocampal theta activity has also been shown to coordinate with activity in the prefrontal cortex (Benchenane et al., 2010) or the ventral striatum (Meer and Redish, 2011) during some spatial memory tasks; this coordination of information processing in multiple brain regions, rather than just locally in the hippocampus, means that all of the information encoded in the different regions for the same phenomenon is bound by the same theta cycle, suggesting that while each theta cycle provides a discrete snapshot of related sensory information about the current conditions at any given moment, information pertaining to different environments is also segregated in time (Fell and Axmacher, 2011; Colgin, 2013). This idea has been further supported by various studies linking individual theta cycles to distinct sets of environmental stimuli or representations of task-relevant maze segments (Gupta et al., 2012). Thus, the theta cycle might facilitate the coordination of inter-areal coding as the hippocampus receives convergent information from multiple brain regions.

Secondly, theta and gamma oscillations may also play a role in synaptic plasticity that underlies learning. *In vitro* and *in vivo* recordings in the rat hippocampus have demonstrated that LTP can be induced when stimulation that otherwise would not induce LTP was paired with the peak of the hippocampal theta rhythm (Huerta and Lisman, 1993; Hölscher et al., 1997). Furthermore, the compression of spike timing via theta phase precession reflects neuronal dynamics that are optimal for hippocampal synaptic plasticity. STDP, a form of plasticity postulated to physiologically model putative synaptic plastic changes during learning, is induced with precise timing (on the order of millisecond

time-scale) of pre- and post-synaptic spikes. While the time differences between sequential activity associated with different place fields is longer than the time-scale of STDP, the temporally compressed activities of place cells with overlapping place fields firing during phase precession mean that temporal separation between spikes within the theta cycle (120 ms) is well suited for the induction of STDP. Thus, cells firing in close temporal proximity could trigger STDP between cells of neighbouring place fields, providing a cellular mechanism for memory storage of the behavioural sequence in the hippocampal network.

Gamma oscillations have also been postulated to play an important role in neural communication and plasticity (Buzsáki and Wang, 2012). Due to its higher frequency, synchronising spikes to particular phases of the gamma cycle ensures a tight temporal regulation and coordination of neural signals, as such, theta and gamma oscillations together work concurrently to support memory functions.

6.4.2 Behaviour

Taking advantage of the cell-type specificity, temporal precision and reversible control offered by optogenetics, I further investigated the effects of cholinergic modulation on various learning and memory processes during different phases of hippocampus-dependent spatial working memory and long-term memory tasks. I found that deviations from an optimal level of neuromodulation can have a detrimental effect on learning. In particular, cholinergic activation and inactivation were both found to impair SLR task performance (Figures 4.6 and 4.7). Indeed, it has been suggested that there are inverted U-shape relationships between cognitive performance and neuromodulatory activity (Yerkes and Dodson, 1908; Cools, 2006) where for a given task demand, there will be an optimal concentration of neuromodulatory output (together with optimal

temporal modulation around this level) that leads to peak performance; neuromodulator concentrations that are larger or smaller than the optimal would produce inefficiencies that may lead to deficits in performance. Thus, the effects of cholinergic activation/inactivation may be beneficial or detrimental depending on basal ACh levels, task demands, and regional differences (Cools, 2006). Further investigation of this relationship with a future dose-response design with various ACh agonists and antagonists will be necessary to confirm this inverted U-shaped hypothesis and may help elucidate the specific contributions of ACh to the cognitive impairments observed in ACh-related disorders such as AD.

The importance of optimal temporal modulation was highlighted in the experiment on the appetitive Y-maze task with ChAT-Ai32 mice where cholinergic activation during memory consolidation slowed learning (Figure 5.2). My working hypothesis is that high levels of ACh during exploration (Giovannini et al., 1998, 2001; Miranda, 2007; Anzalone et al., 2009) promotes theta and gamma activity (Figures 3.4, 3.5 and 3.8), which is important for memory encoding. Lower levels of ACh during subsequent rest and sleep promotes SWRs hypothesised to be important for memory consolidation. Indeed, memory consolidation during consummatory behaviour may have been impaired through ACh-facilitated suppression of SO (Figure 3.9; Vandecasteele et al., 2014). This is supported by findings in which disruption of SWRs by electrical stimulation resulted in learning deficits (Jadhav et al., 2012).

6.4.3 Implications

These findings have important implications for the treatment of patients with cognitive dysfunction because one of the main treatment approaches is the use of AChE inhibitors aimed to increase the levels of ACh in the synapse by preventing their degradation. Given the findings presented in this thesis, it is particularly important to be mindful of the dosage and timing of drug admin-

istration. Indeed, studies have found that low doses of oral AChE inhibitors (MF201, MF268) administered pre-trial could antagonise scopolamine-induced amnesia of spatial memory tasks in rodents, but not in higher doses (Braida et al., 1996, 1998). Similarly, other studies have also found that other cholinergic agonists and cholinesterase inhibitors at low doses also improved task performance but high doses were ineffective (Flood et al., 1981; Wanibuchi et al., 1994; Waite and Thal, 1995). On a cellular network level, the timing of endogenous ACh has been shown to affect the type of synaptic plasticity induced. For example, optogenetic stimulation of septal cholinergic input 100 or 10 ms prior to stimulation of the SC induced an α7 nAChR-dependent LTP or short-term depression, respectively. Conversely, cholinergic stimulation 10 ms after stimulation of SC induced a mAChR-dependent LTP (Gu and Yakel, 2011). Along with the results presented in this thesis, these findings highlight the importance of optimal level of temporally specific cholinergic signalling for encoding of information in the hippocampus. Evaluating whether optogenetic cholinergic activation during encoding could rescue performance deficits observed in AD models could verify the clinical relevance and importance of this temporal precision.

AChE inhibitors are commonly administered orally and as such, their effects largely depend on the rate of absorption from the gastrointestinal tract and and their penetration across the blood-brain barrier. Furthermore, pharmacokinetics is also affected by age and may interact with other drug agents and as such pharmacodynamics can vary from patient to patient, depending on any concomitant treatments that are often common for AD patients (Seritan, 2008; Reichman, 2003). These factors could contribute to the variable results of AChE inhibitors treatments and indicate that achieving an optimal level of cholinergic signalling at specific phases of learning is quite difficult. Therefore, novel therapeutic interventions are necessary for these challenges.

Several recent approaches using brain stimulation have shown promising results in treating neuropsychiatric disorders. Recent findings using transcranial direct current stimulation to modulate brain activation was shown to improve cognitive performance in patients with schizophrenia (Orlov et al., 2017), a symptom domain that is not currently addressed by medication. Deep brain stimulation is another promising electrcal stimulation approach shown to be an effective form of treatment for several disorders. Deep brain stimulation modulate brain activity of specific regions via implanted electrodes. This treatment has been demonstrated to significantly improve motor symptoms in patients with PD (Deuschl et al., 2006). Driven by the need for new effective therapies and the success of recent studies, deep brain stimulation has now emerged as a possible treatment for AD. A recent clinical phase 2 trial (Lozano et al., 2016) revealed that although there was no significant differences in cognitive outcomes for AD patients receiving deep brain stimulation and no stimulation, there was a potential trend for cognitive benefit in older AD patients (>65 years old). Patients were implanted with electrodes in the fornix and the authors indicated that the stimulation may have led to an increase in hippocampus volume. A more sophisticated system that stimulate activation of cholinergic neurons in response to electrical pulses (e.g. ACh release from nanoparticles enclosing ACh following electrical stimulation) could be developed to release an optimal level of ACh with temporal precision. A feedback system to control ACh release depending on the patient's brain state could also be implemented in the future to allow for automatic release of ACh during "appropriate" phases of cognitive function.

6.5 Effect of dopaminergic activation on hippcampal reward place learning

As in previous studies in rodents and monkeys (Tsai et al., 2009; Kim et al., 2012; Stauffer et al., 2016), my findings indicate that optogenetic stimulation of DA neurons during behaviour positively reinforce behavioural measures of value. Furthermore, the results in Figure 5.6 provided one of the first pieces of direct evidence suggesting that DA signalling during a goal-directed learning paradigm can enhance learning. DA has long been recognised to be an important signal in reward learning. Recent findings demonstrated that DA can convert t-LTD into t-LTP before, during and even minutes after the induction protocol (Brzosko et al., 2015), highlighting the power of DA as a positive reinforcement signal to retroactively associate particular experiences with delayed reward during hippocampus-dependent reward learning. Of note, the DA signal presented in Brzosko et al. (2015)'s study was tonic (via bath application of dopaminergic agonists *in vitro*) which is thought to be important for motivational drive and may prime the network to respond appropriately to environmental stimuli (Schultz, 2016). Importantly, the activity of DA neurons can also be phasic and it is the phasic activity that plays a critical role in reward circuits. Phasic bursts of DA activity encode reward prediction errors that coincide with differences between received and expected outcomes (Schultz, 2016). Future experiments with bursts of DA activity to optically induce more phasic-like signal would better validate the findings and evaluate the importance of DA phasic signal in behavioural reinforcement learning in rodents.

Interestingly, while transient DA signal in the absence of food reward was unable to induce goal-directed learning, compared to WT control animals, DAT-Ai32 mice were more motivated to search for the reward (Figure 5.9). This finding further supports the hypothesis that tonic DA is important for motivational drive.

6.6 Cholinergic and dopaminergic modulation of behavioural flexibility

Learning to adapt behaviour to changing environmental conditions is critical for survival and this ability is referred to as behavioural flexibility. At the synaptic level, an attractive mechanism underlying learning and memory is a form of Hebbian learning called STDP. In STDP, the type of plasticity (t-LTP or t-LTD) induced depends on the order and precise timing of pre- and post-synaptic spikes. It is also heavily influenced by neuromodulatory inputs into the hippocampus (Zhang et al., 2009; Brzosko et al., 2015, 2017). Both t-LTP and t-LTD are thought to be required for behavioural flexibility as t-LTP serves to strengthen newly-learnt encoded memory traces while t-LTD is required to weaken earlier encoded memory traces as new information is acquired.

Recent findings have demonstrated that ACh biases STDP to synaptic depression in the hippocampus (Brzosko et al., 2017), visual cortex (Seol et al., 2007) and dorsal cochlear nucleus (Zhao and Tzounopoulos, 2011). In contrast, as mentioned previously, DA promotes t-LTP (Brzosko et al., 2015). This reinforcing signal was also found to convert ACh-facilitated t-LTD to t-LTP (Brzosko et al., 2017). Consequently, Brzosko et al. (2017) proposed a temporally sequential neuromodulation of STDP that enables effective reward-based navigation. They proposed that exploration-associated cholinergic release enhances exploration and when a reward is reached, the synapses of place cells that led to the reward are potentiated via a DA signal. If the reward was not reached at the end of trial, these synapses were instead depressed due to the presence of increased cholinergic tone during exploration. This sequential neuromodulation was postulated to be especially important during reversal learning as the synaptic depression provided by ACh promoted the unlearning of the previously rewarded location, leading to enhanced exploration and, as such, facilitated subsequent learning of the new reward location (Nicholls et al., 2008).

Indeed, my findings support this hypothesis where cholinergic inactivation impairs reversal learning (Figure 5.5) on the OF task but not place learning. I hypothesised that disruption in ACh release during exploration impaired synaptic depression, postulated to be necessary for behavioural flexibility by promoting novel spatial exploration (Kemp and Manahan-Vaughan, 2004; Manahan-Vaughan and Braunewell, 1999), during the task. While this effect may be modest, and therefore undetected during place learning, the disruption of LTD via cholinergic inactivation during reversal learning impaired learning of the new reward location. Indeed, compared to the mice receiving no light stimulation, mice receiving light stimulation throughout the task travelled further to find the new reward location, suggesting an impaired ability to unlearn previously learnt trajectories as previously potentiated synapses were not depressed in the absence of ACh. This is also in agreement with my other proposed finding where DA activation during reversal learning may also impair performance (Figure 5.8). The constant DA signalling could compete with the exploration-associated ACh and thus impair depression; the DA signal throughout the trial may even convert t-LTD to t-LTP, regardless of whether the trajectories lead to the correct or wrong location.

Concurring with my findings, Brzosko et al. (2017) also tested this hypothesis in a computational model. They developed a network model where the location of an agent in the environment is coded by place cells and the speed and direction of the agent are determined by the activity of action neurons that are part of a winner-takes-all network. The synaptic connections between place cells and the action neurons are subjected to the hypothesised sequential neuromodulation of STDP and synaptic weights are updated in all trials. As such, if the agent reaches the reward before the end of a trial, synaptic weights are potentiated, signaled via dopamine. However, if the reward is not found, synaptic weights are depressed, reflecting the effect of increased ACh levels associated with exploration. They found that the presence of both ACh and DA during place learning has a modest improvement compared to simulations that

only potentiate synaptic weights when reward is found at the end of a trial, i.e. DA only, no ACh. Interestingly, similar to my findings, during reversal learning, the absence of ACh leads to an increase in the number of visits to the previous reward location and requires a larger number of trials to learn the new reward location when compared to simulations in the presence of both cholinergic and dopaminergic signalling.

Therefore, the findings in this thesis provided a behaviourally relevant function for the sequential neuromodulation of STDP. It highlighted that in addition to DA (and t-LTP), ACh (and t-LTD) have central roles in effective reward learning, especially in unrewarded trials and for unlearning and the learning of new locations in a familiar environment. Ultimately, chronic recordings of synaptic strengths *in vivo* during learning would be required to conclusively confirm this hypothesis.

6.7 Conclusion and perspective

The work presented in this thesis provides further insights into the role of the cholinergic and dopaminergic systems in various learning and memory processes; namely, it shows that cholinergic activation during reward could impair learning that correlates with an ACh-induced suppression of slow oscillations postulated to be important for memory consolidation. Furthermore, the work suggests that in addition to food reward, transient activation of DA neurons enhances place learning. However, dopaminergic activation alone was insufficient to induce learning in the goal-directed task; yet, these mice were more motivated to search for reward, suggesting an important role for DA in motivation. Finally, the work also presents a behaviourally relevant function for the sequential neuromodulation of STDP proposed in Brzosko et al. (2017) that is especially important for reversal learning. The findings

indicate that cholinergic inactivation and dopaminergic activation appear to impair this process. Together, the work in this thesis highlights the importance of temporally precise neuromodulation and provides some preliminary findings into the different roles that the cholinergic and dopaminergic systems may have in various learning and memory processes.

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